

Government by stealth and secrecy

BRITAIN is now confronted with a severe economic crisis, the ingredients of which are well known, and even when the miners, engine drivers and power station engineers return to full-time working there is bound to be a period of general inconvenience and little prospect of improvements in the standard of living for some time to come. Many things are bound to change as a result of the crisis and it is to be hoped that among them will be the style of government under which Britain has operated for a very long time—specifically the obsessive secrecy and stealth with which Whitehall works and the serious lack of information provided to the public.

This complaint is, of course, fashionable and it is equally fashionable to pay lip-service, as did Mr Heath in 1970, to 'open government'. The question is not whether Britain provides less information than other countries, although it palpably does in comparison with, say, the United States, but whether it provides enough information for those who wish to know, and who do not choose to work for the Civil Service, to be able to come to independent and balanced conclusions.

A conventional defence is to point to such institutions as Question Time in the House of Commons and a free press. This is a very shallow response. The character of Question Time is to find quick, witty and crushing responses to issues raised. This is a political process and success at this facile operation is deemed to be a prerequisite of a good politician. This highly prized skill with words, which spills over into all political exchange, does nothing but damage to a country which needs rational assessment. It is no accident that 'evaluation', a word used almost to excess in the United States, is relatively rare in Britain.

Can a free press make up for this deficiency? Journalists have to depend on a heterogeneous collection of sources for information, from leaks to plain inference. It is simply not possible always to guarantee that one man working against a system will be successful, and it would be foolish to expect more than honest effort. A free press is no replacement for free information and no journalist would regard an increase in the flow of facts as other than beneficial to his operations, certainly not as a threat to his profession.

During the energy crisis the government has operated sufficiently frequently by stealth and suppression of information to raise serious doubts that its style can be supported indefinitely by an electorate. After weeks of denying that the fuel shortage was serious enough to warrant strong corrective action, ration books are issued with such indecent haste that the As and Bs with coughs and colds have probably missed out. After

Post Offices close for the weekend nine days before Christmas an announcement is made that no further Christmas parcels will be accepted. After talking of still going for growth, in the full knowledge of fuel shortages, the government days later imposes a three-day working week and demolishes thoughts of growth.

This is rule by edict and secrecy and is not, it must be emphasised, restricted to the present government. Rather, the channels for advance warning, discussion and compromise fail to exist.

There is much talk at present of the appointment of an energy supremo in Britain, and several names have been mentioned. It is difficult to know whether the idea can be welcomed. If the supremo is a party politician dedicated to the present system of operation and only prepared to give political answers to questions, then the creation of another agency may profit the country not at all. If, on the other hand, someone can be found who will work honestly and openly and recognise the need for the public to receive facts and figures in support of decisions he will deserve wide support.

100 years ago



ON TEMPERATURE CYCLES

SINCE the discovery of an eleven years' period in the phenomena of solar spots, several corresponding periods (it is now well known) have been demonstrated in terrestrial phenomena, more especially in those of magnetism, auroras, cyclones, and rainfall. With regard to weather changes, it has been thought by Dove, that the tracking of a cycle in these could not, theoretically, be made an object of research; and that while some indications of a periodicity might appear, a great part of the complicated changes named must be, from the nature of the case, quite unperiodical. The series of observations by Dove on the subject led him to the conclusion (1) that divergences from the normal, especially those of temperature, are not local, but spread over large surfaces; but (2) that negative divergences, in one region of the earth, are compensated by positive in another; and conversely. That the compensation is perfect, and that the quantity of heat annually given by the sun is constant, has been affirmed also by Maury and others.

From *Nature*, 9, 184, January 8, 1874.



news and views

Global dissemination of hepatitis B virus

INFECTION with the virus, or at least one of the viruses, of hepatitis type B is associated with the appearance in the serum of a specific antigen previously referred to as Australia antigen. Hepatitis B antigen has been found to possess a common group specific surface antigen *a* and at least two principal subdeterminants *d* and *y*, which behave in a mutually exclusive manner although carried on the same antigen particle. Transmission experiments to human volunteers and the common observation of only one subtype in localised outbreaks of hepatitis B infection suggest that the subtypes are the phenotypic expression of distinct genotypes of the virus and are not determined by the host (Le Bouvier, *Am. J. Dis. Child.*, **123**, 420; 1972). Bancroft and his associates (*J. Immunol.*, **109**, 842; 1972) first detected two additional antigenic subdeterminants, named *w* and *r*, and the following phenotypes of hepatitis B virus were recognised: *adw*, *adr* and *ayw*. The missing fourth phenotype *ayr*, which seems to be uncommon, has since been described in the Far East. A remarkable geographical pattern of distribution of hepatitis B antigen subtypes is now emerging (see *Nature new Biol.*, **242**, 34; 1973) with four global zones, where there is an excess of one subtype, and regions where a mixture of subtypes is common.

Diversity of subtypes

Recent studies illustrate this geographical diversity. The high proportion of immigrants in Israel from all over the world provides an almost unique variety of ethnic and geographical background. Approximately half of the blood donors in Israel are foreign-born and most of the others are first-generation Israeli-born Jews. Bar-Shany and her colleagues (*Vox Sang.*, **25**, 105; 1973) characterised 180 asymptomatic antigen-positive blood donors for subdeterminants *d* and *y*: 122 (78%) of 154 males and nine of twelve females carried *y*. Of sixty consecutively identified antigen carriers, selected initially according to their suitability for subtyping and subsequently for representation of dissimilar geographical background, forty-seven (78%) carried the subdeterminant *y* and thirteen (22%) carried *d*. Immigrants to Israel from North Africa, the Near and Middle East, Southern European countries and Southern Russia had almost exclusively subdeterminant *y*, in contrast to the predominance of *d* among blood donors living in North Central and North Europe and North America. Interestingly, Jewish immigrants from South Central Europe have an intermediate frequency of the *d:y* pair between North and South European donors.

Twenty-five hepatitis B antigen-positive samples, selected for suitability for subtyping and for geographical representation, were tested for subdeterminants *w* and *r*. All were found to carry *w*, despite variegated geographical origin. It would be reasonable to anticipate that at least some immigrants may have acquired their antigen carrier state after reaching Israel and therefore that the proportion of *y* may differ from carriers still resident in the countries of origin. Such a shift towards *y* might be expected especially in first-generation Israelis from high *d* prevalence areas, provided

exposure at school or similar places commonly resulted in spread of hepatitis B virus. First-generation Israelis of Northern or Middle European background, however, had the same frequency of subdeterminant *d* as immigrants from these regions, suggesting the home as the important site of transmission.

These facts are in agreement with the view that close and/or prolonged contact is necessary for transmission by hepatitis B carriers. Feinman and his associates (*Lancet*, ii, 867; 1973) subtyped sixty-three symptom-free blood donors, who were found to carry hepatitis B antigen in Toronto: thirty carried subtype *adw*, eight *adr* and twenty-five *ayw*. When the geographical origin of the donors was investigated, the subtypes were found to be unevenly distributed. Most donors who were *adw* were born in Canada, China, Germany and the West Indies, whereas most of those who were *ayw* originated from Mediterranean countries. All eight donors who were *adr* were born in China. 139 family members of forty-six antigen-positive donors were also tested and eighteen (13%) first-degree relatives of nine index cases were found antigen-positive. Subtyping of the antigen in fourteen relatives showed that all had the same subtype as the index case.

The relationship of subtypes to the country of birth and the home rather than place of residence supports the concept that the carrier state of hepatitis B antigen is determined early in life. But even these studies may be an oversimplification of the problem. Soulier and Courouce-Pauty (*Vox Sang.*, **25**, 212; 1973) recently obtained results from subtyping of hepatitis B antigen, using unabsorbed or selectively absorbed antisera, which suggested the subdivision of antigens with the *ay* subdeterminants into three categories and the *ad* subtypes into two categories. These categories could not be explained in terms of the *w* or *r* subdeterminants since *w* was found in all five categories. The results can be explained, however, by postulating the existence of three subdeterminants of the common group specific determinant *a*, so that *a* is designated *a1*, *a2* and *a3*. Thus *ay* is classified as *a1y* (found in this study in 13% of apparently healthy Vietnamese carriers), *a2y* (found in most healthy French carriers and patients with antigen subtype *ay*) and *a3y* (mostly African carriers of *ay*). On the other hand, *ad* is subdivided into *a2d* (common *ad*) and *a3d* (apparently a very rare subtype).

The differences between the subspecificities of *a* may be both qualitative and quantitative, probably with overlapping and predominance between these three subdeterminants. As with the *d* and *y* subdeterminants *a1*, *a2* and *a3* are related to the virus and not to the host. In this study it was noted, for example, that whereas *a1* was found in Vietnamese, it may also be found in Caucasians after a stay in Vietnam; conversely, some Vietnamese living in France were found to carry *a2*. The same is true for *a2*, which is common in Caucasians (but not restricted to them) and, although *a3* seemed to be most common in African countries, it was also found in French Caucasians who became infected in Africa.

Geographical zones

The overall picture which is therefore emerging is a Y zone, with antigen phenotype *ayw*, incorporating the Middle East, Iran and Pakistan, the Eastern Mediterranean and Southern European countries and Africa (although Africa has not been adequately studied). The D zone, with phenotype *adw*, is centred in North Europe, North and Central America,

and both *adw* and *adr* are prevalent in the Far East, Thailand, Malaysia, Indonesia and New Guinea. The R zone, predominantly *adr*, seems to be located in South East Asia and the Far East. The fourth region is the Pleotypic zone, centred in Oceania and particularly around Bougainville, as reported on page 38 of this issue of *Nature* by Mazzur and her colleagues. In this region practically all possible mixtures of the recognised phenotypes of hepatitis B antigen have been found.

Incursions of different subtypes into these zones and the establishment of mixed zones inevitably result from population movements. But since the subtypes are determined by the virus and not by the host, an epidemic of one strain of hepatitis B virus might result in a spate of that particular uniform strain at one time, and a later epidemic of a different subtype might leave carriers of another subtype even in the same area. This has been recorded in Stockholm, for example. Magnus and his associates (*Scand. J. infect. Dis.*, 5, 81; 1973) examined stored sera from seventy-two patients with viral hepatitis admitted to a hospital in Stockholm in 1953–54 and sera from 202 patients with hepatitis admitted in 1970. Hepatitis B antigen was detected in 43% and 49% of the patients respectively. The subtypes from these two groups and from ninety-five antigen-positive drug addicts were determined. Twenty-two (71%) of thirty-one patients from the 1953–54 group were of *ad* subtype whereas only two were *ay* (seven samples could not be typed). In 1970, however, seventy-five (76%) of ninety-nine patients were *ay* whereas only twelve were *ad* (the remaining twelve sera could not be subtyped). Ninety (95%) of the antigen-positive drug addicts were *ay* and five were *ad*. A shift has thus occurred in Stockholm since 1953–54 from predominantly *ad* to the *ay* subtype. It is speculated that the tourist influx from Northern Europe to the Southern Mediterranean countries, the Middle East and North Africa, an *ay* zone, as well as the attraction of these countries to drug addicts may have been factors in the shift of hepatitis B antigen subtypes noted in Stockholm.

The latest report by Mazzur and her associates in the current issue of *Nature* also confirms the varied geographical distribution of antigen subdeterminants *d*, *y* and *w*, and, although the antigen mapping of the world is fragmentary and far from complete, information is rapidly accumulating. Hepatitis B antigen has previously been shown to be a useful epidemiological marker; it may well prove to be a fascinating ecological marker of both ancient and more recent population migration.

From our Medical Virology

Correspondent

Plant diversity in grassland

from our Plant Ecology Correspondent

THE publication by the Kent Trust for Nature Conservation of papers given at a conference on chalk grassland management held a year ago at the King's College Field Centre, Rogate, Hampshire, will reopen the question of what determines plant species diversity in this habitat (see *Nature*, 240, 443; 1972). It may also raise new questions regarding the objectives of management practices and priorities in conservation.

The diversity issue has been clarified by Grime (*Nature*, 242, 344; 1973), who has come to the conclusion that diversity in grassland habitats is clearly related to environmental stresses. If stress is low, then grasslands become dominated by species of high physical competitive ability, that is species which grow rapidly, are robust and produce dense litter. Stress reduces the performance of these species, allowing other species to enter the community as a result of the lowering of competition. Diversity thus increases with stress until the stress becomes so severe that only species which are specifically adapted to that particular adverse factor are capable of survival. Then diversity falls. Some authors emphasise the role of predation in the maintenance of vegetation diversity (for example, Harper, *Brookhaven Symp. Biol.*, 22, 48; 1969) whereas others feel that nutrient availability may be the critical factor (for example, Green, *Biol. Conserv.*, 4, 378; 1972).

In the recently published report (*Chalk Grassland*, ed. by Jermy and Stott, Kent Trust for Nature Conservation; 1973), both sides of the argument are represented. Wells considers that the composition of a grassland community can best be understood by reference to the respective life forms and growth periods of its constituent species. In other words, if mowing or grazing stresses are applied to a grassland then the species which lose the smallest proportion of their above ground biomass will be at a selective advantage. Tall, robust species are eliminated to the advantage of short-growing species, thus allowing the development of a diverse assemblage of species with an average 67% of low-growing hemicryptophytes.

Harding defends the point of view that nutrient availability limits the growth of aggressive species. Using Shannon's diversity index, he demonstrates a negative correlation between angiosperm diversity in the grass sward and total nitrogen in the soil. He follows Green in the belief that a build-up of nitrogen in the soil permits invasion and attainment of dominance by robust species, thus reducing overall diversity. It is always dangerous, how-

ever, to infer causation from correlation. One could equally well argue that the presence of robust, dominant species causes a build-up of nitrogen in the soil by rapid litter production and hence the encouragement of soil microbiol activity, including nitrogen fixation. Suspension of robust species by applying a grazing stress could keep soil nitrogen levels low. The nutrient argument still lacks direct experimental support.

The quest of conservationists for biotic diversity becomes even more complex when one considers the zoological aspect of the problem. For invertebrates in particular, microclimatic diversity (which is a function of vegetation structural complexity) is an overriding influence upon the potential faunal diversity. It is not surprising, therefore, that Morris demonstrates a positive correlation between the number of species of *Auchenorhyncha* and the vegetation height at the sampling sites. It does, however, pose a management problem, since in selecting a management regime which determines vegetation height, one must choose between floral and faunal diversity.

Proteins and odour perception in insects

from our Insect Physiology Correspondent

THERE have been several suggestions that enzymatic processes are involved in olfactory perception in insects. Schneider observed that the antennae of the male silkworm rapidly degrade the sex pheromone bombycol following stimulation; and Kasan and Kaissling confirmed that bombycol, (E)-10-(Z)-12-hexadecadien-1-ol, was progressively degraded into acidic and ester fractions by enzymes in the antennae and other parts of the body. In this way the receptors quickly become competent once more to receive the olfactory stimulus. Norris, Ferkovich and others have shown that naphthoquinones, which function as feeding inhibitors in the cockroach *Periplaneta*, exert their activity by binding to sulphhydryl groups of specific antennal proteins.

Ferkovich, Mayer and Rutter (*J. Insect Physiol.*, 19, 2231; 1973) have now studied the sex attractant of the cabbage looper, *Trichoplusia ni*, along the same lines. This pheromone is (Z)-7-dodecen-1-ol acetate. When exposed to a soluble protein present in the male antennae it suffers enzymatic conversion to the alcohol (Z)-7-dodecen-1-ol—which not only lacks sex attractancy but is a potent inhibitor of behavioural responses to the normal pheromone. The protein responsible for enzymatic hydrolysis of the pheromone exists in smaller amounts elsewhere in the body, but it is most active in the antennae. The inhibitory

alcohol also reacts, non-enzymatically, with the antennal protein; and both these interactions seem to be necessary for olfactory perception by the cabbage looper.

The close analogies between olfaction and juvenile hormone activity have been pointed out (*J. Insect Physiol.*, **15**, 73; 1969). The hormone likewise must be rapidly inactivated if it is to be kept under control; and the chief mode of inactivation is by means of an esterase which hydrolyses the terpenoid ester to the carboxylic acid, which is devoid of hormonal activity.

KCN and photosystem one

from our Photosynthesis Correspondent

THERE now seems to be no doubt that KCN inhibits photosynthesis by specifically blocking electron flow through photosystem one (S1). It has long been recognised that KCN will inhibit photosynthesis but its mode and site of action have not been clearly understood. Izawa *et al.* (*Biochim. biophys. Acta*, **314**, 328; 1973) now convincingly demonstrate that this compound inhibits electron transfer between cytochrome *f* (cyt *f*) and P700.

Izawa *et al.* used differential spectrophotometry and electron paramagnetic resonance (EPR) to monitor changes in the redox states of these two components which are closely associated with S1 activity. Oxidation of the S1 reaction centre can be detected either by an absorbancy change at 700 nm (for this reason it is called P700) or as an EPR signal with a *g* value of 2.0025. Cyt *f* acts as an electron donor to P700 and is oxidised by light preferentially absorbed by S1 (S1 light). When photosystem two (S2) light is superimposed on S1 light the oxidation levels of cyt *f* and P700 are reduced. These were classical observations first made in Duysen's laboratory in Holland some years ago which clearly demonstrated that S1 and S2 act in series and that cyt *f* and P700 are on the reducing side of the S2 water splitting reaction centre. As expected it was found that in the presence of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU), a compound which blocks electron flow from S2 to S1, the reduction of oxidised cyt *f* and P700 by S2 light was inhibited.

Izawa *et al.* were able to demonstrate these properties in their spinach chloroplast preparations, but when the chloroplasts were incubated with KCN, S1 light only photo-oxidised P700 and not cyt *f*. Moreover, when cyt *f* was chemically oxidised the action of S2 light was to reverse this oxidation. On the other hand KCN severely restricted the net flow of electrons from S2 to photo-oxidised P700. These observations indi-

cate without doubt that KCN acts by blocking electron flow between cyt *f* and P700. Izawa and colleagues argue that KCN interacts with plastocyanin, as suggested from *in vitro* experiments, and that this copper containing protein is the intermediate electron carrier between cyt *f* and P700.

Their general ideas have additional support from experiments involving the use of the artificial electron donating agents, diaminodurene, reduced 2,6-dichlorophenol indophenol and reduced N-methylphenazonium methosulphate (PMS) all of which bring about the reduction of oxidised cyt *f* and P700 in DCMU insensitive reactions. It was found with these artificial systems that KCN usually inhibited the reduction of P700 but not cyt *f* although leak past the KCN block was obtained in some conditions such as high PMS concentrations (>30 μ M). Unlike normal electron flow this component did not support photophosphorylation.

This work of Izawa *et al.* not only gives good support to an old argument that cyt *f* is not the primary electron donor to S1 but also demonstrates that KCN can be used as a new and powerful tool for investigating S1 reactions in isolated coupled chloroplasts.

Satellites advance geodetic research

from a Correspondent

UNDER the auspices of the International Association of Geodesy, an important symposium on the Earth's gravitational field and secular variations in position was held in Sydney from November 26 to 30.

Through the Earth and Ocean Physics Applications Project (EOPAP) of NASA, the GEOS-C is due to be launched in 1974, and will utilise many of the techniques evolved through the ranging reflectors placed on the Moon by the Apollo astronauts. Many of the papers presented at the symposium described techniques for use with such satellites as elevated targets for ranging with pulsed lasers. The astonishingly high accuracy possible with these new methods will shortly become standard expectations in surveying and in geophysical and oceanographic research.

In an invited presentation, P. V. Angus-Leppan (University of New South Wales) assessed the future prospects for "four-dimensional geodesy", a term which describes the new high-precision techniques for providing position and gravity, and their changes within a short time span. He pointed out that the accuracies of one part in 10^8 , which are being approached in these measurements, will shortly allow direct measurements of the velocity of movement of continental plates; from

geological inference these plates are currently believed to be moving at a rate of about a few centimetres per year. To achieve the required accuracy, a network of observing stations must be set up on a global scale with international coordination. Angus-Leppan said that each station would constitute the fundamental geodetic observatory for its region and it would include, in a set of comprehensive facilities, laser ranging systems to both the Moon and artificial satellites, an antenna for interconnecting stations by very long baseline interferometry (VLBI), Doppler satellite instruments and a base for absolute gravity measurements.

Progress in the VLBI technique was discussed by several contributors. P. F. MacDoran (Jet Propulsion Laboratory, California Institute of Technology) reported that observations of intense extragalactic radio sources (notably QSOs) with two separated dish antennas operating at S-band wavelengths (13 cm) gave accuracies of a few centimetres for three-dimensional surveying over short separations of 16 km. In addition to measuring variations in universal time, intercontinental baselines (8,400 km) have been measured in VLBI tests between Goldstone, California, and Madrid, Spain. MacDoran predicted that, if a transportable 9 m diameter dish antenna were operated in conjunction with a fixed dish, like the 64 m ones at Goldstone and Madrid, accuracies of a few centimetres in three-dimensional positions would be practical for separations of up to several hundred kilometres, using X-band wavelengths (4 cm).

The importance of accurately calibrated measurements of position and gravity with time was emphasised by the geophysically-minded delegates. These measurements lead to estimates of slow, yet important, lateral and vertical velocities of crustal plates. Direct tests will then be possible for recent theories for predicting earthquakes related to dilatancy of rocks. Four Americans (D. E. Smith, R. Kolenkiewicz, R. W. Agreen and P. J. Dunn) described SAFE which has recently started to use satellite ranging techniques for measuring the motion between two points 900 km apart on opposite sides of the San Andreas fault system in California. Over a 7-yr period, the experimenters believe they will determine the average relative motion of the two sides of the fault to an accuracy of about 5 mm yr⁻¹.

The geoid is defined as the equipotential surface of the Earth's gravitational field corresponding to 'mean sea level', but, as R. S. Mather (University of New South Wales) pointed out, geodetic levelling results and mean sea level (as defined by tide gauge readings) do not correspond. The 'mounds' in the sea, with amplitudes of up to 2 m and wave-

lengths as great as 8,000 km, produce a topography of the sea surface. This leads to problems in geodesy because of the reliance on mean sea level as a reference datum for elevation. Mather indicated ways of solving this problem by using the precise radar altimeters in the satellites of the forthcoming EOPAP project. If confirmed and when mapped on a global scale, the topography of the sea surface will provide information for predicting ocean currents—a potential boon for more economic navigation of commercial shipping.

J. D. Boulanger (Institute of Physics of the Earth, Soviet Academy of Sciences) tackled the complex problem of the observed variations in gravity with time, and the interpretation of these data in terms of, for example, tectonic processes occurring within the Earth's interior. Yet, according to Boulanger, significant data on this point are scanty. So he proposed an international programme for studying the precise variation of gravity with time throughout the world. He described a 10-yr programme being established in the Soviet Union with a network of gravimetric stations of high precision in the territory of Eastern Europe and the west of the Asiatic part of the Soviet Union.

The network will be based on Sèvres, near Paris. Sèvres has been chosen by the Russians because, Boulanger said, this is the only place where an absolute result for a rate of change of gravity with time has been obtained. The experimenter responsible for this result, A. Sakuma (Bureau Internationale des Poids et Mesures) described the determinations of absolute gravity made by his team; the precision of their measurements is almost one part in 10^9 .

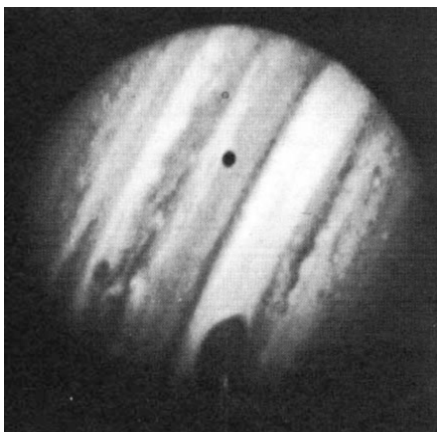
Pioneer 10 exceeds expectations

THE remarkable pictures of Jupiter received from Pioneer 10 are alone sufficient to ensure that the mission is remembered as a success, even without data from the other experiments on board the spacecraft. It will take some time for details of these data to emerge; but the simple fact that Pioneer 10 passed unscathed through Jupiter's magnetosphere is itself enough to require understanding of the planet's radiation belts to be rethought.

Most astronomers expected the spacecraft to suffer some damage when it encountered energetic particles trapped in the Jovian magnetosphere, and some suggested that the experiments and telemetry would be rendered completely useless. But a few voices suggesting the opposite view were raised, and two papers in the issue of *Science* dated December 7 (four days after Pioneer 10's closest approach to Jupiter) show the

extremes of opinion which co-existed before the spacecraft's encounter with the planet. Stansberry and White describe (*Science*, **182**, 1020; 1973) a model of the trapping of electrons and protons from the solar wind by the magnetic field of Jupiter which implies very high particle densities in the radiation belts, reaching levels 100 times greater than those used in the design of Pioneer 10. "This indicates", they said in the paper, "that there is a significant chance of radiation damage to the spacecraft".

Clearly, they were wrong. And a clue to just how their calculations were in error comes from a study by Hess, Birmingham and Mead (*Science*, **182**, 1021; 1973) of the effect of Jupiter's satellites on the particle fluxes in certain parts of the Jovian magnetosphere. Three of the Galilean satellites, in particular, turn out to be very effective in



Jupiter's red spot, a shadow of the Moon Io and Jupiter's cloud structure are shown in this photograph taken on December 1 as Pioneer 10 was about 2,500,000 km (1,580,000 miles) from the planet.

limiting the fluxes of energetic protons and electrons diffusing inward. Ganyমেদে, Europa and Io all produce "precipitous" drops in the flux, so that the average flux in the plane in which these satellites lie "should be about a factor of 100 less than . . . if there were no absorbing moons". Hess and colleagues suggest that "This may be enough to prevent serious radiation damage to the spacecraft", and it now looks as if they were right.

The importance of this, of course, extends beyond the satisfaction of having an idea proved right. It seems clear from the success of Pioneer 10 that spacecraft can approach fairly close to Jupiter without being severely damaged, and the presence of a satisfactory theory to account for the low particle fluxes encountered by the spacecraft suggests that similar low flux conditions will be encountered by other spacecraft following suitable trajectories. The need for close encounters is two-fold: first, it enables spacecraft to take full advan-

tage of Jupiter's gravity in directing them on to Saturn and the other gas giants; second, with the present state of the art a Jupiter rendezvous mission, leaving a spacecraft in orbit around the planet, can only be achieved by firing the spacecraft's motors when it is deep in the Jovian gravitational well. So Pioneer 10 has lived up to its name by showing the way for future missions to the outer reaches of the Solar System.

Code of practice for radiation protection

from a Correspondent

A MEETING in London on November 14 was arranged jointly by the British Institute of Radiology and the Hospital Physicists' Association to provide a forum for discussion of the requirements and implementation of the 1972 revision of the Code of Practice for the Protection of Persons against Ionising Radiations arising from Medical and Dental Use.

The morning session was concerned with the main provisions of the new code and difficulties in its interpretation. J. Cole (Dudley Road Hospital, Birmingham) reviewed the steps leading to the present revision following the publication of the first edition of the code in 1957. He pointed out that the basic policy in formulating the code was "to set out the basic principles, and to give general guidance on good practice".

H. Miller (University of Sheffield) described the changes in the scope of the code and the general protection measures required. He said that the wording of the new code was more precise and the conditions for both essential and desirable requirements had been clarified.

G. M. Ardran (University of Oxford) and S. K. Stephenson (Christie Hospital, Manchester) then outlined the changes in the code relating respectively to the medical and physical aspects of diagnostic radiology. The relative responsibilities of the clinician and radiologist in reducing patient exposure were mentioned in relation to the problems of implementing the '10-day rule' for avoiding irradiation of unsuspected pregnancies. Stephenson enumerated some of the twenty-five new obligatory requirements in the code; he felt that reduction of patient dose was the area where improvements could be made.

The measures for increasing protection in radiotherapy were examined by T. J. Deeley (Velindre Hospital, Cardiff); again a more positive identification of hazards was evident in the new code. Leakage radiation from therapy equipment and diaphragm systems were unsatisfactory features leading to unnecessary patient irradiation.

That the section of the code dealing

with unsealed radioisotopes had required and received drastic revision was explained by N. G. Trott (Royal Marsden Hospital, Sutton), although he pointed out that the current revision had not fully kept abreast of recent changes in nuclear medicine technique. N. J. D. Smith (King's College Hospital, London) surveyed a large number of practices and revealed many with many unsatisfactory protection standards. Personnel monitoring had shown consistently high exposures to many dentists and ancillary staff, who were often unaware of the existence of the code of practice. It was reported that few dentists had taken advantage of a recent monitoring scheme launched by the National Radiation Protection Board. The need for a separate, explanatory supplement to the code for dentists was evident.

The afternoon session was devoted to problems in implementing the code, and the achievement of radiation safety in the hospital service. D. C. Holdsworth (United Hospital, Sheffield) thought that clinicians could make an effective contribution in reducing patient exposure, but they needed more information in order to weigh the risks of the radiation procedure against the hazards of omission. C. K. Warrick (Royal Infirmary, Newcastle upon Tyne) described his system for implementing the '10-day rule' in a survey 12 months ago; the rule was practised by only 20% of the departments sampled. Several hospitals had demonstrated that workable systems could be operated to reduce the number of unsuspected pregnancies irradiated.

The responsibilities of the Radiation Protection Adviser in achieving radiation safety were discussed by R. F. Farr (Queen Elizabeth Hospital, Birmingham), and N. Chesney, Farr's colleague, made a plea for a clear statement of the duties of the Radiological Safety Officer and, in many cases, appropriate training for these duties was required.

W. J. Meredith (Manchester Regional Hospital Board) spoke in the unusual guise of administrator, and pointed out that the code put a heavy responsibility on the controlling authority. Apart from the need to keep the numerous formal records required by the code, there was a continuing need to keep radiation safety committees alive and active.

R. D. Moore (Weston Park Hospital, Sheffield) described his attempts to evaluate the cost of protection measures. The annual cost in the United Kingdom of health physics services in the hospitals together with the costs of structural shielding amounted to some £580,000 per year. This resulted in a consequential saving of 140,000 man-rads, that is about £4 per man-rad saved.

Coulomb-nuclear effects

from our Nuclear Theory Correspondent

AN interesting example of interference between Coulomb and nuclear scattering amplitudes has recently been found in the inelastic scattering of heavy ions by nuclei. This interference can be understood classically in a qualitative way, and can also be calculated accurately using the distorted wave theory.

This interference is made possible by the particular character of the field between heavy ions, which allows incident particles with different classical impact parameters to be scattered through the same angle. To see how this comes about, consider the behaviour of the scattering angle as the impact parameter is steadily reduced.

For large impact parameters the projectile interacts only with the repulsive Coulomb field of the target, and is deflected through a small angle that steadily increases as the impact parameter is reduced. When the distance of closest approach becomes comparable to the sum of the radii of the projectile and target, the nuclear force begins to attract the projectile, thus reducing the scattering angle. At still smaller impact parameters the projectile is repelled by the centrifugal force, thus increasing the scattering angle again. The resulting variation of scattering angle is shown in Fig. 1 for 60 MeV ^{16}O ions on ^{58}Ni .

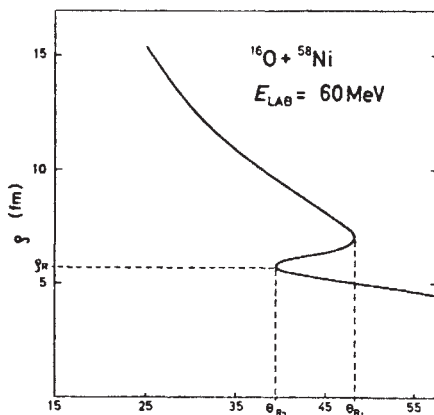


Fig. 1 Variation of the scattering angle θ with the impact parameter ρ for 60 MeV ^{16}O ions on ^{58}Ni (Malfliet, Landowne and Rostokin, *Phys. Lett.*, **44B**, 238; 1973).

In such cases there are three values of the impact parameter corresponding to each scattering angle between the critical scattering angles $\theta(R_1)$ and $\theta(R_2)$. The chief contribution to the scattering comes from angles around the critical angles, so a semi-classical theory can be made by adding the corresponding amplitudes, with appropriate phase and attenuation factors. The turning point corresponding to the larger impact parameter refers to the orbit further

away from the nucleus where the projectile is moving mainly in the Coulomb field, whereas the other turning point refers to the orbit nearer the nucleus where the projectile is under the influence of both the Coulomb and nuclear fields. These amplitudes interfere, and because they vary with angle in a different way the interference is sometimes destructive and sometimes constructive.

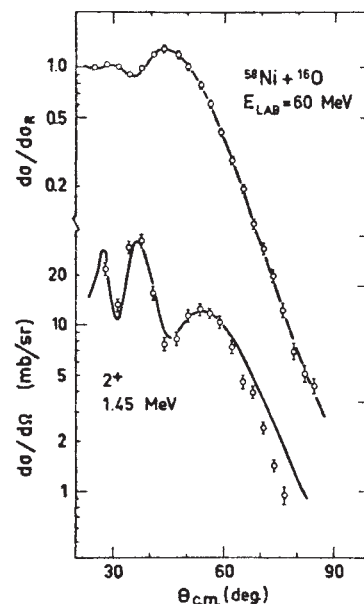


Fig. 2 Differential cross sections for the elastic and inelastic scattering of 60 MeV ^{16}O ions by ^{58}Ni showing the interference effects in the region of the critical angles. The curves are obtained using the distorted wave theory (Christensen, Chernov, Gross, Stokstad and Videbaek, *Nucl. Phys.*, **A207**, 433; 1973).

These effects appear in elastic scattering around the region of the critical angles, but are much more prominent in inelastic scattering. For small angles, the cross section for the excitation of the lowest $J^\pi=2^+$ state of the target is almost entirely due to Coulomb excitation, while for large angles it is mostly due to nuclear excitation. In the intermediate regions around the critical angles the interference effects are strongly marked as shown in Fig. 2.

The whole process can be described quantitatively by the distorted wave theory, using the appropriate vibrational model for the 2^+ state. The results of such calculations are also given in Fig. 2 and show that the interference effects are accurately given by the theory. Detailed fitting in the interference region gives improved values of the parameters of the distorting potential and of the nuclear dynamical deformation parameter.

This interference phenomenon is a good example of the way interactions between heavy ions can be understood semi-classically and then analysed by the distorted wave theory to give additional information on nuclear structure.

Two Stage Red Sea Floor Spreading

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Evidence is presented which strongly suggests that most of the southern Red Sea is underlain by oceanic crust which evolved in two stages, the first from about 41 to 34 million years ago and the second from about 4 to 5 million years ago to the present day.

THE Red Sea consists of a main trough and a young axial trough which starts at about 24° N and widens towards the south¹. Unfortunately, large amounts of sediment obscure the true structures especially in the southern half. It has been clear for some time that the axial trough is underlain by new oceanic crust but there are conflicting views on the nature of the crust underlying the main trough¹⁻¹⁴.

The scant amount of gravity data available in the 1950s was sufficient to show that the axial trough is underlain by a high density intrusive zone¹⁵. Further geophysical work confirmed the presence of this intrusive zone¹. Large magnetic anomalies with amplitudes exceeding 1,000 nT were observed over the axial trough and seismic velocities of about 7 km s⁻¹ were found at shallow depths of 2 to 3 km. The magnetic anomalies were later interpreted in terms of seafloor spreading and a half rate of 1 cm yr⁻¹ was determined for latitude 16° N (ref. 3). It became clear that the Red Sea axial trough is being formed by seafloor spreading, and Arabia and Nubia have been drifting apart for the past 3 to 4 Myr.

The situation regarding the main trough and earlier history of the Red Sea has been much less clear. The fact that in many places the shelves are at or near sea level suggested in the 1950s that they are underlain by Precambrian shield rocks¹⁵. At that time the large thicknesses of sediments^{9,16} were unknown. Further, seismic velocities of 5.9 km s⁻¹ were found¹ suggesting the presence of at least some shield rocks. In the mid 1960s, the evidence (previously disputed) for about 100 km shear along the Dead Sea rift became very strong¹⁷. This 100 km displacement implies that there must be a much greater separation of the Red Sea than the width of the axial trough². Seismic refraction experiments at 22 to 23° N with the objective of finding the extent of the oceanic crust^{4,8} showed an average velocity of 6.63±0.16 km s⁻¹ at a mean depth of 4.6 km for the main trough, thus establishing the presence of oceanic crust and a minimum separation of Nubia and Arabia of 130 km. But some recent papers^{12,14,18} still support the thesis that only the narrow axial trough is oceanic and the main trough and shelves are underlain by downfaulted shield rocks.

New evidence is given here which supports the existence of oceanic crust beneath the shelves of the southern Red Sea. In addition, it is now possible to be reasonably certain about the age of this crust.

New Magnetic Data

In 1966, the Gulf Oil Company flew a detailed total intensity magnetic survey at a height of 610 m over the western margin of the Red Sea between 14.5 and 16° N; and in 1968, US Project Magnet flew eight lines (four at height 305 m and four at height 3,658 m) from shore to shore at 18° N.

The Gulf survey has excellent navigation control, Shoran being used. The original analogue records have recently been made available and have been digitised with the object of gaining information on the seafloor spreading history of the margins. The profiles were digitised at every Shoran location and additionally at every turning point so as to preserve the details of the records. This gave a digitisation interval of about 0.7 km. A ground magnetometer station was set up at Massawa and the records from here were used to correct for the daily variation. The IGRF 1965.0 gradient was removed from each profile.

For the Project Magnet profiles, Doppler navigation was used and hence these have less good positional control. The records were digitised in a similar way and the IGRF 1965.0 gradient removed.

Nine of the sixty Gulf profiles (G11, G16, G20, G25, G31, G35, G40, G44 and G50) and two of the eight Project

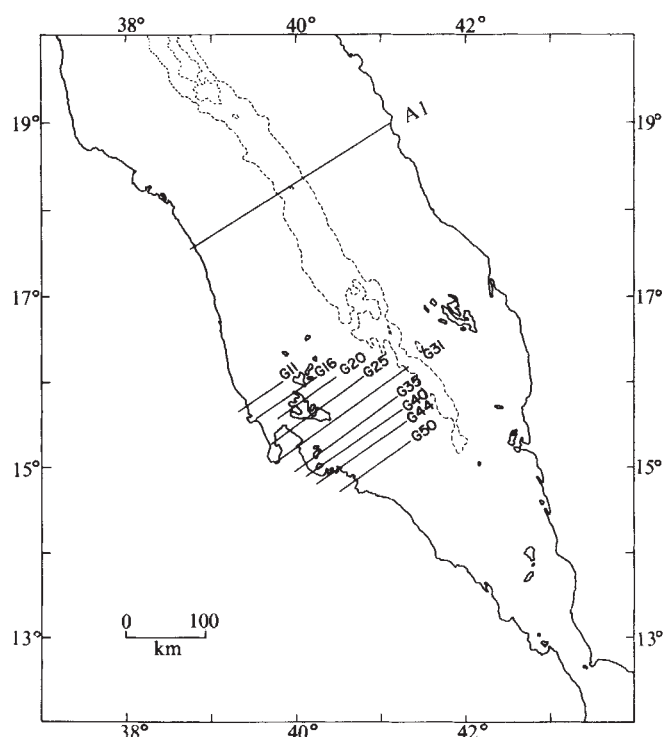


Fig. 1 Location of total intensity magnetic profiles. The dashed lines are the 500 and 1,000 fathom contours taken from the 1 : 2 million bathymetric chart of Laughton²⁵.

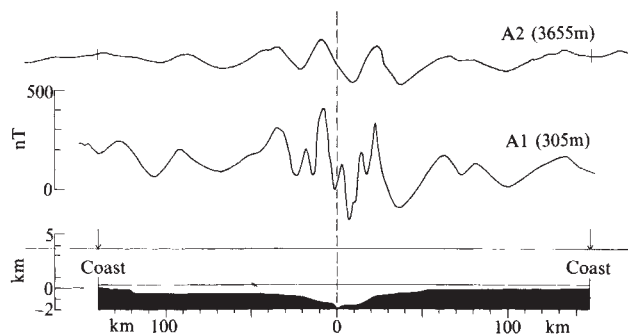


Fig. 2 Project Magnet profiles across the whole width of the Red Sea at 18° N showing the existence of magnetic anomalies on either side of the axial trough associated with symmetrical spreading (compare Fig. 5) and their relationship to the bathymetry.

Magnet profiles (A1 and A2) are used to illustrate the sea-floor spreading history of the Red Sea. The locations of the profiles are shown in Fig. 1. A2 (height 3,655 m) has almost the same location as A1 (height 305 m).

Interpretation of Magnetic Anomalies

Figures 2 and 3 show two sets of examples of magnetic anomalies over the Red Sea. Figure 2 has two profiles flown at different heights from shore to shore (Project Magnet) showing the relationship of the anomalies to the bathymetry. Figure 3 shows a set of nine profiles over the western margin (Gulf Oil Co.). Taken together, these provide convincing evidence for the presence of oceanic crust beneath nearly all of the southern Red Sea.

First, the Project Magnet profiles (Fig. 2) show the familiar large anomalies over the axial trough and also the existence of anomalies over the main trough. It should be remembered

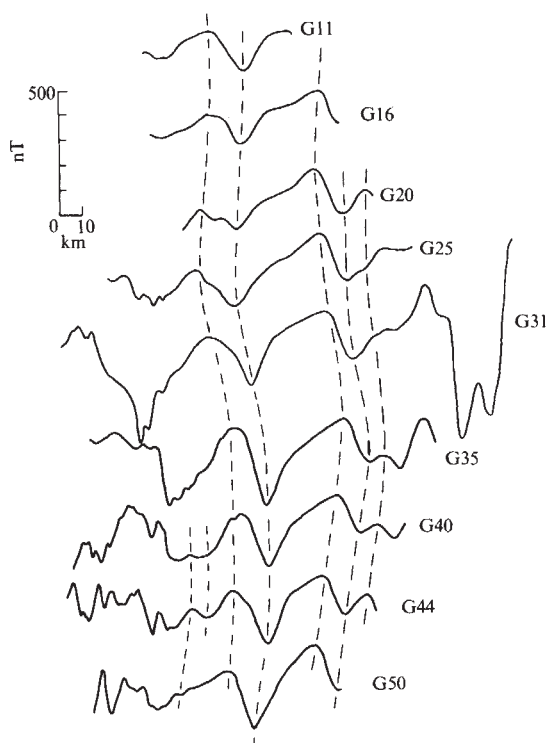


Fig. 3 Nine profiles from the Gulf magnetic survey illustrating the two types of anomalies, the smooth long wavelength well correlated anomalies over the Red Sea and the poorly correlated high frequency anomalies in the west.

that the computed anomalies for a normally magnetised body with the northwesterly strike of the Red Sea show a positive anomaly over its western edge and a negative over its eastern edge (a reversely magnetised body has a negative over the western edge and a positive over the eastern edge). When consideration is given to this, it is apparent that the anomalies are due to symmetrically emplaced bodies (Fig. 5) and the symmetry is impressive evidence that the anomalies are due to seafloor spreading. Figure 2 also shows a contrast in the amplitude of the anomalies over the axial trough and those over the margins suggesting two different phases of spreading. Further, the large anomalies are not exactly confined to the axial trough but extend somewhat further. Possible reasons for this are discussed by Girdler and Whitmarsh¹⁹.

Second, the Gulf profiles (Fig. 3) show that the anomalies on the western side of the axial trough are remarkably consistent and can be linearly correlated over a distance of at least 230 km. This would be very unusual for anomalies over shield rocks and provides additional evidence for their sea-floor spreading origin.

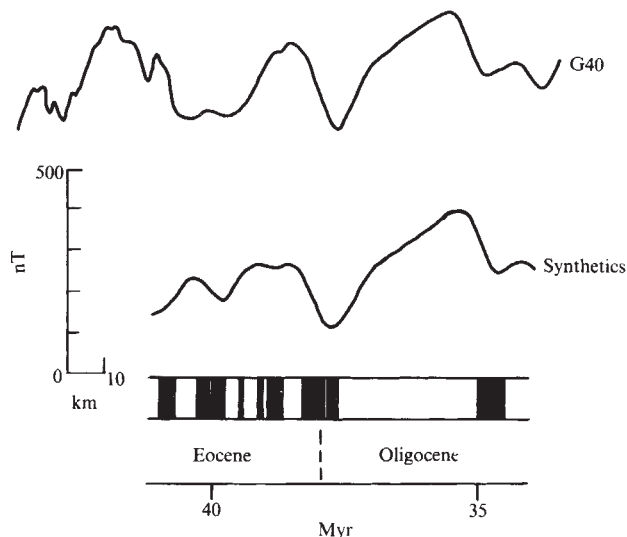


Fig. 4 Synthetic and observed profiles for Gulf profile G40. The synthetic profile is for a spreading rate of 1.4 cm yr⁻¹ and strike N 120°.

Third, the Gulf profiles (G25, 31, 35, 40, 44 and 50) show a contrast between two types of anomalies—high frequency poorly correlated anomalies in the west and long wavelength well correlated smooth anomalies in the east over the Sea. Almost certainly the former are over Precambrian shield and the latter over seafloor.

Age of Red Sea Floor

Synthetic profiles were generated for the Red Sea using the reversal time scale of Heirtzler *et al.*²⁰. At first, attempts were made to obtain an interpretation similar to that for the Gulf of Aden²¹, that is, a continuous spreading history back to anomaly 5 (10 Myr). The correlations beyond 3 to 4 Myr were found to be very poor, as found by Vine³. The synthetic profiles were then extended back to the base of the Miocene (25 Myr) using various spreading rates and again good correlations could not be found. Finally, synthetic profiles were generated back to 70 Myr and searched for possible correlations with the observed anomalies.

A remarkably good correlation was found for the time interval 34 to 41 Myr. This is illustrated in Fig. 4 using Gulf profile G40. A spreading rate of 1.4 cm yr⁻¹ over this

time interval gives excellent correlations with the observed data. The long reversal interval (35.00 to 37.61 Myr) is necessary to obtain the characteristics of the Gulf profiles. Other possible candidates for this long reversal interval are even older (for example, 49.58 to 52.41 Myr) but for these the fits for the neighbouring anomalies are not nearly so good. It is therefore proposed that the oceanic crust to the sides of the axial trough is surprisingly old, being about 34 to 41 Myr (late Eocene to early Oligocene).

New Model for the Red Sea

A new geophysical model for the Red Sea is presented in Fig. 5. This utilises the shore to shore Project Magnet profile A1, the location of which is shown in Fig. 1. Various data such as thickness of sediments and seismic refraction profiles suggest that the old crust is at a depth of about 5 km and the new crust at about 2 km. The synthetic magnetism is compared with the observed. The fit of the anomalies for the older crust is not quite so good as for the Gulf data and this is considered to be partly due to the poorer navigation. The old phase is shown to end at about 34 Myr and the new phase to begin at just over 4 Myr. There is, of course, some uncertainty about these dates. It seems highly probable that the new phase of spreading may have commenced at the end of the Miocene (4.9 Myr ago²²) when the whole sedimentary environment changed with the opening of the southernmost Red Sea to the Indian Ocean.

Support for the two stage model also comes from seismic reflection profiling^{6,18}. The acoustic reflector (S) is now known from Deep Sea Drilling to be anhydrite and to represent the top of the Miocene²³. This reflector is absent in the axial trough suggesting a second phase of spreading after the Miocene.

The model of Fig. 5 is in broad agreement with the heat flow seafloor spreading models of Sclater and Francheteau²⁴. The axis of the Red Sea has very high heat flow consistent with the generation of new oceanic crust and the heat flow over the sides is slightly high⁹ consistent with older oceanic crust.

Plate Tectonics

Figure 6 depicts a map of the Red Sea (Mercator projection) based on the 1:2 million bathymetric chart of Laughton²⁵ and showing the 500 and 1,000 fathom contours. The ages of the oceanic crust deduced from the magnetic

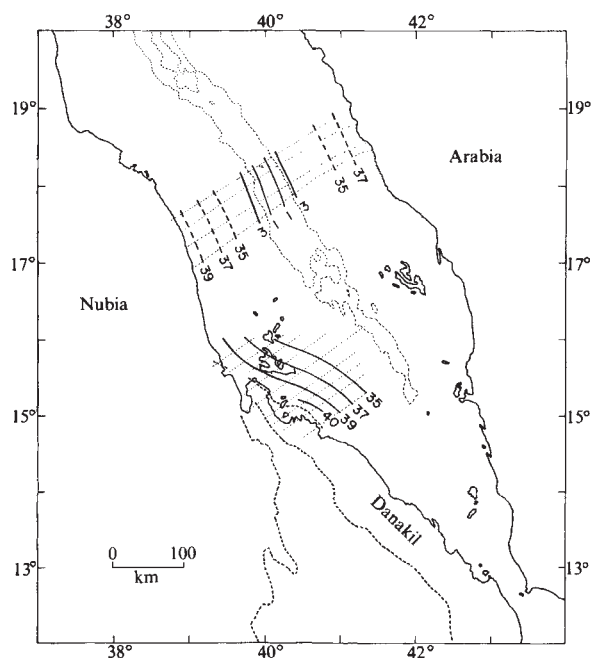


Fig. 6 Isochrons (Myr) for the ages of oceanic crust beneath the Red Sea. The dashed lines are less certain than the solid lines. The dotted lines are flight tracks.

profiles have been transferred to the map. For the Project Magnet data (18° N) the isochrons for the new phase of spreading are fairly easily correlated over all the profiles. It is less easy to correlate the anomalies for the old phase of spreading especially on the eastern side and the 35, 37 and 39 Myr isochrons are thus shown with dashed lines.

From the Gulf magnetic profiles it is possible to locate the 35, 37 and 39 Myr isochrons with some confidence and to tentatively mark the oceanic/continental boundary of the Danakil plate. The isochrons bend southeastwards in this region. This is presumably because the Danakil plate was rotating counterclockwise with respect to the Arabian plate during this early phase of spreading. The anomalies can be seen to be closer towards the south (Fig. 3) especially for profiles G25 to G50. This is to be expected if Danakil was rotating with respect to Arabia during this time interval. To the north, away from Danakil, the anomalies are related to the counterclockwise rotation of Arabia with respect to Nubia.

Geological History of the Red Sea

In view of the evidence given here for an early phase of seafloor spreading, it is necessary to present a new summary of the geological history of the Red Sea and check that the model given in Figs 5 and 6 is consistent with the geological data. In particular, the geological summary²⁶ given by Girdler needs substantial revision (Fig. 7). The most important change is in the date of formation of the main Red Sea graben previously given as end of Oligocene/early Miocene, that is, about 26 Myr.

In the geological review given by Heybroek²⁷, it is reported that some kind of depression may have existed as early as Carboniferous times. The first major rift movements seem to have been in the lower/middle Eocene. Following these, a major phase of seafloor spreading occurred in the late Eocene and early Oligocene. This was followed by a long quiet period of about 30 Myr during which time huge thicknesses of sediments, mainly evaporites, were deposited.

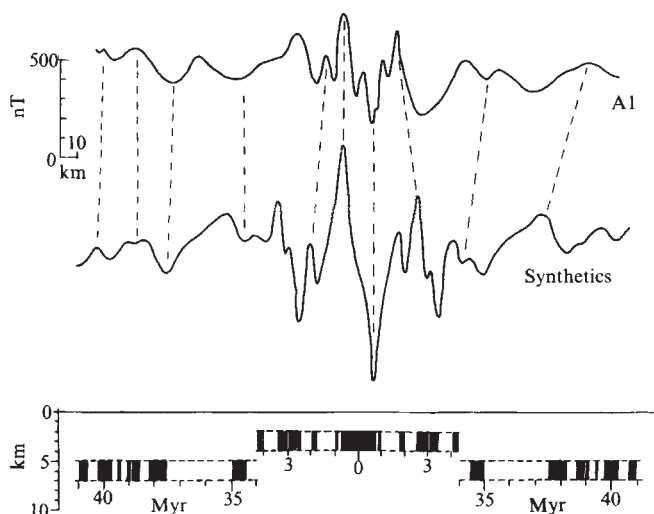


Fig. 5 A new geophysical model for the Red Sea (18° N) with two phases of seafloor spreading, the first (1.3 cm yr^{-1}) being in the late Eocene/early Oligocene and the second (0.9 cm yr^{-1}) being over the past few million years and still continuing.

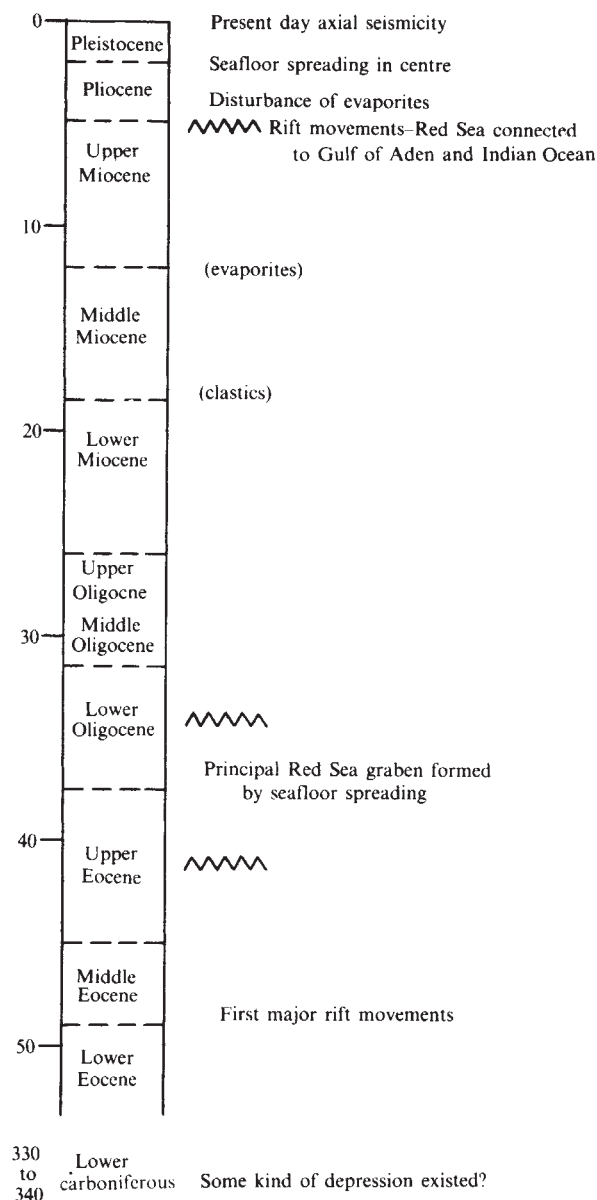


Fig. 7 Geological history of the Red Sea.

From oil company data¹², the thickness of bedded evaporites is estimated to be as much as 5 km. Some K/Ar dates are available¹³ from exploration wells in the south-west Red Sea (about latitude 16° N). These are 36.4 ± 2 Myr and 36.6 ± 2 Myr for the Amber well bottomhole core consisting of slightly metamorphosed very pyritic argillite and an average of 30 Myr for the bottomhole basalt core of well B1. These ages are no longer embarrassing, for it is now evident that the early stage of the Red Sea was in existence at least 40 Myr ago.

After the long 30 Myr quiet period of deposition, a new period of activity started about 5 Myr ago. The whole sedimentary environment suddenly changed at the end of the Miocene; nanno oozes were deposited instead of evaporites as the Red Sea became connected to the Gulf of Aden and Indian Ocean via the Straits of Bab-el-Mandeb. Seafloor spreading recommenced along the centre giving rise to large magnetic anomalies and very high heat flow. The axis of the Red Sea is seismically active and the process is still continuing²⁸. The axial deep trough (Fig. 2) is due to the separation of old oceanic crust plus overlying sediments. The new oceanic crust beneath the axis reaches to within 2 to 3 km of sea level because of its higher temperature.

The new phase of spreading caused considerable disturbance of the sediments with the creation of a very unstable situation as the crust and sediments parted and the axial trough evolved¹⁹.

Some Implications

Finally, some implications concerning the possible existence of two stage Red Sea floor spreading are given.

The first concerns the Dead Sea rift. Freund *et al.*²⁹ find evidence for about 105 km shear, the whole of which can be dated as post-Cretaceous (about 65 Myr). This movement took place in two stages, the last 40 to 45 km being post-Miocene/early Pliocene. This correlates well with the second (recent) phase of spreading in the Red Sea. It has not yet been possible to date the remaining 60 km of shear²⁹ and it now seems likely that this was late Eocene/lower Oligocene corresponding to the first major phase of spreading in the Red Sea. It is to be hoped that Middle Eastern geologists will look for evidence to confirm this.

The second concerns the Gulf of Aden. Since little or no shear has been observed along the Ethiopian rift (between the Nubia and Somalia plates) it is to be expected that the history of the Gulf of Aden should be similar to that of the Red Sea. The interpretation of continuous seafloor spreading over the past 10 Myr in the Gulf of Aden²¹ depends largely on the correct identification of anomaly 5 (not identified in the Red Sea). The magnetic profiles are therefore being re-examined to see if the two stage Red Sea spreading model also applies to the Gulf of Aden. Whitmarsh (personal communication) points out that as anomaly 5 is correlated into the north-west Indian Ocean, this is also in question. It may be that magnetic profiles over the oceans should be carefully re-examined for evidence of go-stop-go seafloor spreading.

Lastly, the Afar region (bordered by the Nubia, Somalia and Danakil plates) has been a popular location for a mantle hot spot. If hot spots provide the driving mechanism for plates, the evidence presented here suggests that this hot spot was active about 40 Myr ago, died for 30 Myr and relit about 5 Myr ago! This seems somewhat unlikely and it seems more likely that the intermittent plate motions of Arabia, Nubia, Danakil and Somalia are a consequence of more mantle wide convective motions.

We thank the US Naval Oceanographic Office and the Gulf Oil Co. for making available the original magnetometer records and for giving us permission to publish the profiles shown here. In particular, we acknowledge the help of Drs Henry Stockard and Patrick Taylor (USNOO) and Drs J. P. Huie (vice-president), A. C. Armstrong, M. D. Beltrandi and A. Pyre (Gulf). Stuart Hall helped with the digitisation of the Project Magnet records.

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¹ Drake, C. L., and Girdler, R. W., *Geophys. J. R. astr. Soc.*, **8**, 473 (1964).

² Girdler, R. W., *Geol. Surv. Canada Pap.*, **66-14**, 65 (1966).

³ Vine, F. J., *Science, N.Y.*, **154**, 1405 (1966).

⁴ Tramontini, C., and Davies, D., *Geophys. J. R. astr. Soc.*, **17**, 225 (1969).

⁵ Abdel-Gawad, M., *Phil. Trans. R. Soc.*, **A267**, 23 (1970).

⁶ Phillips, J. D., and Ross, D. A., *Phil. Trans. R. Soc.*, **A267**, 143 (1970).

⁷ Allan, T. D., *Phil. Trans. R. Soc.*, **A267**, 153 (1970).

⁸ Davies, D., and Tramontini, C., *Phil. Trans. R. Soc.*, **A267**, 181 (1970).

⁹ Girdler, R. W., *Phil. Trans. R. Soc.*, **A267**, 191 (1970).

¹⁰ Hutchinson, R. W., and Engels, G. G., *Phil. Trans. R. Soc.*, **A267**, 313 (1970).

¹¹ McKenzie, D. P., Davies, D., and Molnar, P., *Nature*, **226**, 243 (1970).

¹² Lowell, J. D., and Genik, G. J., *Bull. Am. Assoc. Petrol. Geol.*, **56**, 247 (1972).

¹³ Girdler, R. W., and Darracott, B. W., *Comm. Earth Sci. Geophys.*, **2**, 131 (1972).

- ¹⁴ Hutchinson, R. W., and Engels, G. G., *Bull. Geol. Soc. Am.*, **83**, 2989 (1972).
- ¹⁵ Girdler, R. W., *Q. Jl geol. Soc. Lond.*, **114**, 79 (1958).
- ¹⁶ Frazier, S. B., *Phil. Trans. R. Soc.*, **A267**, 131 (1970).
- ¹⁷ Freund, R., *Geol. Mag.*, **102**, 189 (1965).
- ¹⁸ Ross, D. A., and Schlee, J., *Bull. geol. Soc. Am.* (in the press).
- ¹⁹ Girdler, R. W., and Whitmarsh, R. B., *Rep. Deep Sea Drilling Project*, **23** (in the press).
- ²⁰ Heirtzler, J. R., Dickson, G. O., Herron, E. M., Pitman, W. C., and Le Pichon, X., *J. geophys. Res.*, **73**, 2119 (1969).
- ²¹ Laughton, A. S., Whitmarsh, R. B., and Jones, M. T., *Phil. Trans. R. Soc.*, **A267**, 227 (1970).
- ²² Gill, J. B., and McDougall, I., *Nature*, **241**, 176 (1973).
- ²³ Shipboard Scientists, *Geotimes*, **17**, 24 (1972).
- ²⁴ Sclater, J. G., and Francheteau, J., *Geophys. Jl R. ast. Soc.*, **20**, 509 (1970).
- ²⁵ Laughton, A. S., *Phil. Trans. R. Soc.*, **A267**, 21 (1970).
- ²⁶ Girdler, R. W., in *Hot Brines and Recent Heavy Metal Deposits in the Red Sea* (edit. by Degens, E. T., and Ross, D. A.), 38 (Springer-Verlag, 1969).
- ²⁷ Heybroek, F., in *Salt Basins around Africa*, 17 (Institute of Petroleum, 1965).
- ²⁸ Fairhead, J. D., and Girdler, R. W., *Phil. Trans. R. Soc.*, **A267**, 49 (1970).
- ²⁹ Freund, R., Garfunkel, Z., Zak, I., Goldberg, M., Weissbrod, T., and Derin, B., *Phil. Trans. R. Soc.*, **A267**, 107 (1970).

Isolation of Bovine Thymin: a Polypeptide Hormone of the Thymus

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Two closely related polypeptides termed thymin I and II were isolated from bovine thymus by their effect in causing a delayed impairment of neuromuscular transmission *in vivo*. Purified thymin also induced bone marrow cells to develop the characteristics of intrathymic lymphocytes and it is probable that thymin is a lymphopoietic differentiating hormone of the thymus with secondary neuromuscular effects.

THE thymus is a compound organ consisting of an epithelial stroma derived from the third branchial arch and lymphocytes derived from stem cells originating in haemopoietic tissues (see ref. 1 for review). Lymphocytes differentiated within the thymus leave as mature thymus-derived cells (T cells) which circulate to the blood, lymph, spleen and lymph nodes. The induction of stem cell differentiation within the thymus is probably mediated by secretions of the epithelial cells of the thymus² but difficulties with bioassays have hindered the complete isolation and structural characterisation of these hormones³⁻⁶.

A different approach to the isolation of thymic hormones was developed from experimental studies related to the human disease myasthenia gravis. Myasthenia gravis is characterised by a deficit in neuromuscular transmission and an associated thymic pathology has long been recognised in this disease. A study of clinical, pathological and immunological features of myasthenia gravis led to the hypothesis that thymic autoimmune disease is the lesion basic to this disease^{7,8}. A laboratory animal model of experimental autoimmune thymitis was developed and these animals were shown to have an abnormality of neuromuscular transmission similar to that of myasthenia gravis by a number of criteria⁹⁻¹⁴. Experimental analysis of this laboratory model revealed that the effect on neuromuscular transmission was mediated by a substance released from the diseased thymus^{9,13,14}. Neurophysiological studies in thymectomised and thymus grafted animals suggested that this substance

was secreted physiologically¹⁵; and so the name thymin was proposed for this putative hormone.

The hypothesis of a neuromuscular blocking substance in the thymus is not novel; it was proposed in 1943 (ref. 16) in relation to the then current concepts of the curare-like nature of the myasthenic neuromuscular block, the known association of thymic pathology with the disease and the empirical discovery that thymectomy was an effective treatment for myasthenia gravis^{17,18}. An unequivocal demonstration of such a neuromuscular blocking substance was not, however, obtained despite a large body of experimental work (reviewed in ref. 1). Although no consistent acute effects of thymus extracts could be demonstrated it was found that when thymus extracts were injected into animals impairment of neuromuscular transmission could be detected after a delay of several days¹⁹. This effect of a delayed production of impairment of neuromuscular transmission has served as a bioassay for monitoring the chemical fractionation of bovine thymus extracts.

I shall describe the isolation of two closely related polypeptides which produce, following a single injection of 4-32 ng per mouse, an impairment of neuromuscular transmission detectable by electromyography 1-5 d following injection. Of particular interest is the finding that these purified polypeptides also induce differentiation of bone marrow cells to T cells and hence seem to be thymic hormones which induce differentiation of the lymphopoietic stem cells which give rise to thymus-derived lymphocytes (T cells) (R. S. Basch and G. Goldstein, in preparation).

Assay Systems

Extracts were injected into test animals and neuromuscular transmission was studied after an interval of 1-5 d. Initially 100 g guinea pigs were injected intraperitoneally; neuromuscular transmission was studied *in vitro* with a preparation of the phrenic nerve and hemidiaphragm¹⁹. Subsequently 200 g rats were used; fractions were injected subcutaneously and neuromuscular transmission was assessed electromyographically¹². Finally, mice were found to be an effective test system. Female Swiss-Webster mice weighing 25-35 g were injected intraperitoneally, groups of five to ten mice being used to test each fraction. The animals were anaesthetised with urethane and electromyography was performed as described previously¹¹. The muscle action potentials were recorded in each mouse following supramaximal nerve

Mice were given 100 ng of thymine I or solvent and five mice from each group were tested at various times. A statistically significant ($P < 0.001$) effect on neuromuscular transmission was first detected 18 h after injection, became maximal by 24 h and persisted through 5 d (Fig. 3).

Relationship of Thymine I and Thymine II

During the development of the isolation procedures for thymine, the purity of fractions was assessed by polyacrylamide disc electrophoresis at pH 8.9. Neuromuscular blocking activity was found associated with material which stained with Coomassie blue, and had an R_F of 0.30 with respect to bromophenol blue (Fig. 4). At pH 8.9 thymine I migrated only slightly faster than thymine II. At pH 4.3, however, this material resolved into two distinct bands both of which produced neuromuscular impairment (see above). The faster one was designated thymine I (R_F 0.63 with respect to methyl green) and the slower one thymine II (R_F 0.57) (Fig. 4). The mobilities of these purified polypeptides remain consistent and do not interconvert.

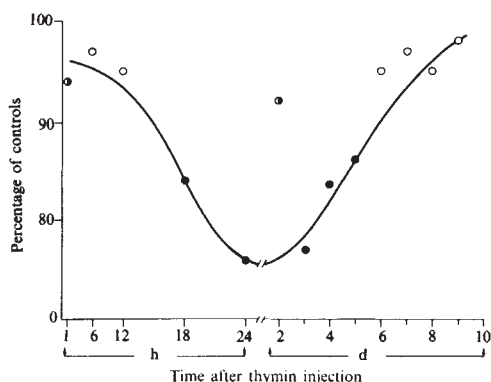


Fig. 3 Electromyographic response of mice at various times after a single injection of 100 ng thymine I expressed as a percentage of the response of mice injected with solvent alone. With supramaximal nerve stimulation at 50 impulses s^{-1} the height of the tenth muscle action potential was expressed as a percentage of the first. This change was calculated for five points in each of five to ten mice from each group. For each time point the change in thymine-treated mice was expressed as a percentage of the change in solvent-treated control mice and the statistical significance of the difference was assessed by Student's t test; solid circles: $P < 0.001$; half solid circles: $0.05 > P > 0.001$; open circles: $P > 0.05$.

Thymine I and II are polypeptides with molecular weights of approximately 7,000, based on their characteristics in molecular sieve chromatography and their amino acid compositions. Preparations of thymine I and II were pure by the criterion of polyacrylamide disc electrophoresis. With 200 μ g of thymine I or II per gel there was a single major band stained with Coomassie blue in gels run at pH 8.9 or pH 4.3. Analysis of the terminal amino acids provided further evidence for purity. No residue could be identified at the N terminus of thymine I or II which was therefore considered blocked. Only lysine was identified at the C terminus of both thymine I and II (D. S. Schlesinger, G. Goldstein and H. D. Niall, in preparation).

Thus thymine I and thymine II seem to be very similar. They were closely associated during the various fractionation procedures and it was difficult to establish chromatographic conditions that would resolve them. The presence of at least some areas of common primary amino acid sequence was suggested by two sets of observations. Thymine I and thymine II were digested with trypsin and the tryptic peptides were mapped in two dimensions by chromatography and high voltage electrophoresis. Several peptides with similar positions were found in thymine I and thymine II, suggesting common sequences. Rabbit antisera raised against either

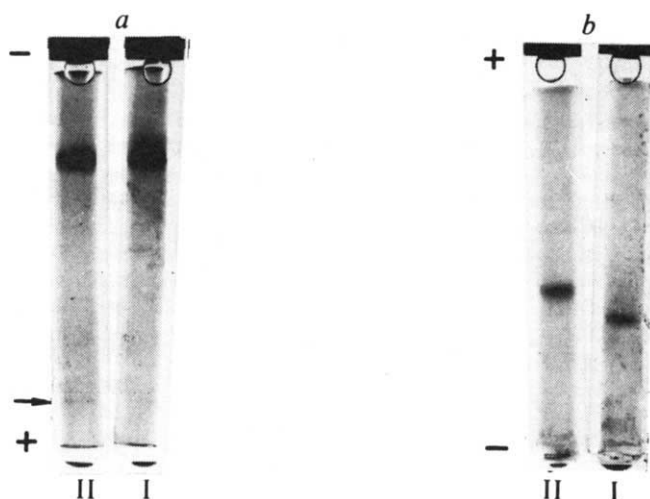


Fig. 4 Polyacrylamide gels from disc electrophoresis of 200 μ g samples of thymine I and thymine II, a at pH 8.9 and b at pH 4.3. The gels were fixed with trichloroacetic acid and stained with Coomassie blue. One major band is present in each gel. Arrow indicates dye marker.

thymine I or thymine II reacted with both and in two dimensional immunodiffusion on micro-Ouchterlony plates thymine I and thymine II produced a line of identity (Fig. 5). This again provided evidence that thymine I and thymine II had a common tertiary configuration being recognised by the antibodies and so probably had some common primary sequence.

Yet there is a consistent biochemical difference between thymine I and II as detected by ion exchange chromatography at pH 10.5 and polyacrylamide disc electrophoresis at pH 4.3. Although the evidence summarised above suggests that thymine I and II share extensive primary sequence it is still uncertain whether one represents a chemically modified form of the other or whether they are two distinct polypeptides

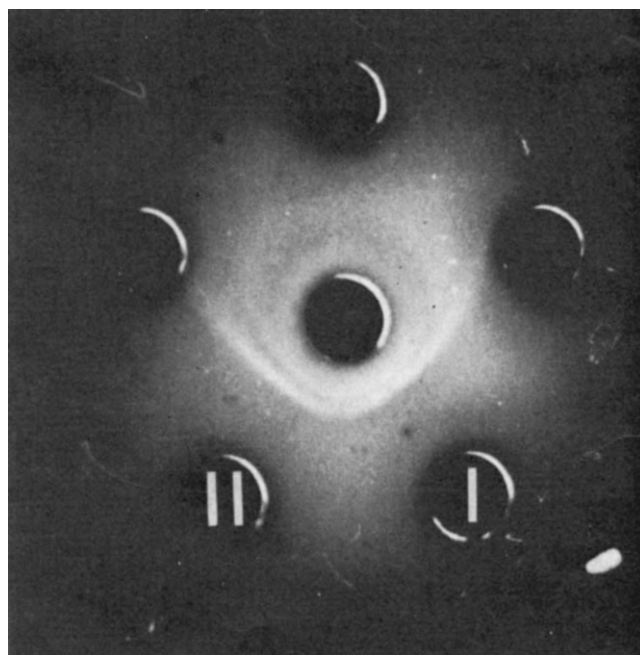


Fig. 5 Micro-Ouchterlony immunodiffusion with antiserum to thymine I in the centre well, thymine II (1 mg ml^{-1}) in the lower left well and thymine I (1 mg ml^{-1}) in the lower right well. After 24 h diffusion the plate was fixed in trichloroacetic acid and stained with Coomassie blue for photography.

with close sequence homology based on a common ancestral gene.

Biological Significance of Thymins

An interpretation of the findings in myasthenia gravis as suggestive of autoimmune thymitis^{7,8} led to the development of a model of the disease in laboratory animals, experimental autoimmune thymitis⁹⁻¹⁴. Experimental analysis of this model implicated a substance released from the diseased thymus in the pathogenesis of the neuromuscular lesion^{9,13,14} and experiments on thymectomised and thymus-grafted animals indicated that this substance was also secreted by the normal thymus¹⁵. This thymic substance affecting neuromuscular transmission was termed thymin.

The prime function of the thymus is the differentiation of lymphopoietic stem cells originating in the haemopoietic tissues to mature thymus-derived cells (T cells) capable of involvement in the immune response of the body^{2,6}. Since this differentiating function is probably mediated by a hormone secreted by the epithelial cells of the thymus the finding of a hormone affecting neuromuscular transmission raises two possibilities. The thymus may secrete two separate hormone systems, one differentiating lymphopoietic stem cells, the other affecting neuromuscular transmission. Alternatively, by virtue of some similarity in the receptors of lymphopoietic stem cells and the neuromuscular synapse, the one hormone may affect both.

Purified thymin I, in subnanogram concentrations, caused a detectable neuromuscular lesion and also induced the differentiation of bone marrow cells *in vitro* in that they acquired antigens characteristic of intrathymic T cells (R. S. Basch and G. Goldstein, manuscript in preparation). Thus these findings suggest that thymin(s) are primarily hormone(s) of the thymus, inducing differentiation of lymphopoietic stem cells and that the effect on neuromuscular transmission is secondary and may be related to the presence of some receptor in the synaptic apparatus which is similar to that of the stem cells. Cholinergic receptors are present on both stem cells²¹ and mature lymphocytes²², and it is possible that the receptors of stem cells which induce differentiation to T cells may have evolved from such cholinergic receptors.

By this reasoning the neuromuscular effect of thymin is a chance one, rather than a primary physiological regulating function of the body. Nevertheless, the mode of action of thymin in producing neuromuscular block is of great interest. Although thymin results in a postsynaptic block in neuro-

muscular transmission¹² the evidence is that it does not combine directly with the classical acetylcholine receptor since it is inactive *in vitro*¹⁹, causing neither acute blocking nor depolarisation, and it has an 18 h latent period *in vivo*, the effect then persisting for several days. These findings would suggest that thymin is affecting a regulatory mechanism in the neuromuscular synapse and thymin may be a valuable tool for probing such a mechanism of chronic modulation of synaptic conductivity.

This work was supported by the Irvington House Institute and grants from the National Institute of Neurological Diseases and Stroke of the US Public Health Service, the Muscular Dystrophy Associations of America and the Sackler Research Fund. Ronald King and Miriam Siegelman provided technical assistance. I am grateful for the advice and collaboration of Drs H. N. Niall, D. S. Schlesinger, R. S. Basch and M. E. Lamm.

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- ¹ Goldstein, G., and Mackay, I. R., in *The Human Thymus* (Heinemann, London, 1969).
- ² Goldstein, G., *Vox Sang.*, **19**, 97 (1970).
- ³ Goldstein, A. L., Guha, A., Zatz, M. M., Hardy, M. A., and White, A., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1800 (1972).
- ⁴ Small, M., and Trainin, N., *J. exp. Med.*, **134**, 786 (1971).
- ⁵ Bach, J. F., and Dardenne, M., *Cell. Immun.*, **3**, 11 (1972).
- ⁶ Komuro, K., and Boyse, E. A., *Lancet*, **i**, 740 (1973).
- ⁷ Goldstein, G., *Lancet*, **ii**, 1164 (1966).
- ⁸ Goldstein, G., *Clin. exp. Immun.*, **2**, 103 (1967).
- ⁹ Goldstein, G., and Whittingham, S., *Lancet*, **ii**, 315 (1966).
- ¹⁰ Goldstein, G., and Whittingham, S., *Clin. exp. Immun.*, **2**, 257 (1967).
- ¹¹ Goldstein, G., and Hofmann, W. W., *J. Neurol. Neurosurg. Psychiat.*, **34**, 453 (1969).
- ¹² Goldstein, G., and Manganaro, A., *Ann. N.Y. Acad. Sci.*, **183**, 230 (1971).
- ¹³ Kalden, J. R., Williamson, W. G., Johnston, R. J., and Irvine, W. J., *Clin. exp. Immunol.*, **5**, 319 (1969).
- ¹⁴ Kawanami, S., and Mori, R., *Clin. exp. Immunol.*, **12**, 447 (1972).
- ¹⁵ Goldstein, G., and Hofmann, W. W., *Clin. exp. Immunol.*, **4**, 181 (1969).
- ¹⁶ McEachern, D., *Medicine, Baltimore*, **22**, 1 (1943).
- ¹⁷ Blalock, A., Mason, M. F., Morgan, H. J., and Rivers, S. S., *Ann. Surg.*, **110**, 544 (1939).
- ¹⁸ Keynes, G., *Br. J. Surg.*, **33**, 201 (1946).
- ¹⁹ Goldstein, G., *Lancet*, **ii**, 119 (1968).
- ²⁰ *Chemical Formulations for Disc Electrophoresis* (Canalco, Rockville, Maryland, 1968).
- ²¹ Byron, J. W., *Nature new Biol.*, **241**, 152 (1973).
- ²² Strom, T. B., Deisseroth, A., Morganroth, J., Carpenter, C. B., and Merrill, J. P., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2995 (1972).

Structure of Yeast Phosphoglycerate Kinase

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A 3.5 Å resolution electron density map of yeast phosphoglycerate kinase has been calculated from which a skeletal model of the enzyme's single polypeptide chain has been constructed. The position of the nucleotide substrate ADP when bound to the enzyme has been defined.

WE recently reported the structure of yeast phosphoglycerate kinase (PGK) as seen at 5 Å resolution¹. This work and that of Blake *et al.*², who have carried out similar crystallographic studies with horse muscle phosphoglycerate kinase, have shown that this monomeric enzyme is composed of two lobes connected by a waist region. A 3.5 Å resolution electron density map of the yeast enzyme has now been calculated, from which it is possible to follow the course of the polypeptide chain starting in one lobe and passing through the central waist to form the structure of the second lobe before returning to complete the structure of the first lobe.

The reaction catalysed by PGK is the first of the two ATP-forming reactions of the glycolytic pathway. By soaking crystals of the enzyme in solutions containing nucleotide substrates we have also located the position of a unique Mg-ADP binding site near the surface of one of the two lobes.

Structure Determination

Two single-site heavy atom derivatives of PGK were obtained by soaking crystals with mercuric acetate or sodium aurichloride respectively, as previously described¹. X-ray intensity data to 3.5 Å resolution were collected from the native protein using a computer-controlled four-circle diffractometer. These 7,820 measurements were divided into sets on the basis of intensity, each set containing approximately one-tenth of the X-ray reflections. Data for each of the two derivatives were collected for the six sets of highest intensity, totalling 3,752 independent Bijvoet pairs. Scaling of derivative to native data and the refinement of heavy atom parameters were carried out separately for each set of reflections. Anomalous scattering data were included in refinement and phase calculation procedures. The mean figure of merit³ for all measured reflections was 0.76. The electron density distribution of the native enzyme, calculated using centroid phases⁴, was plotted on Cobex sheets, in sections of constant y on a scale of $10^3:1$. A molecular model was built using Nicholson model components (Lab-quip, Reading, England), which were fitted to the electron density using an optical comparator⁵. In the absence of sequence information, each amino acid residue was inserted as L-alanine.

Interpretation of Electron Density

For much of its length, the course of the polypeptide chain could be followed without difficulty, and density corresponding to the larger amino acid side chains was clearly visible. Helical regions of the polypeptide chain were also immediately apparent, as is shown in Fig. 1. Since the correct 'hand' of the protein had been determined using the anomalous scattering data¹, the polarity of the chain could be defined by noting the orientation of the side chains in helices and in some runs of extended chain. As expected at this resolution, the main chain carbonyl oxygens were not resolved from the rest of the main chain, so that the orientation of the peptide bonds could not be determined directly. The planar peptide groups were therefore adjusted to give the best fit to the main chain density, and to locate β -carbon atoms at side chain density where this was clearly indicated. At bends in the chain, it was sometimes impossible, in the absence of sequence information, to tell how much of the density was due to main chain and how much to side chain atoms, so that an amino acid may have been omitted or inserted in such places. Also, the close approach of several runs of chain makes it difficult to determine, by immediate inspection, the correct connection between strands entering and leaving two small inadequately resolved sections of the molecule. The correct interpretation must produce for the molecule as a whole a single, continuous polypeptide chain with each element of the secondary structure having the correct polarity. This criterion was used to resolve any ambiguity in difficult regions of the map.

Knots in the polypeptide chain would not be expected to arise during spontaneous folding of the protein, but might easily be produced in a model by misinterpretation of the electron density map. In this case it is possible to trace the entire course of the chain starting from the amino terminus without having to pass the growing carboxyl end through any loop of chain already produced. It must be emphasised, however, that, apart from any possible error in the X-ray measurements, the absence of sequence information and the possible existence of flexible sections of polypeptide chain make certainty of interpretation unattainable at this stage

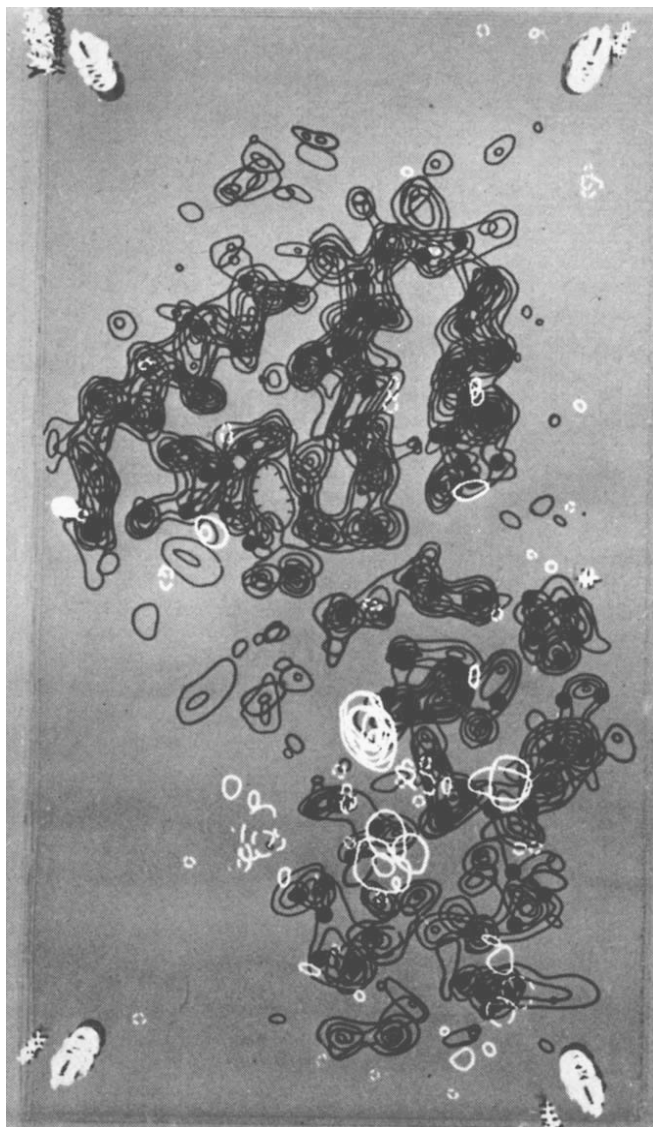


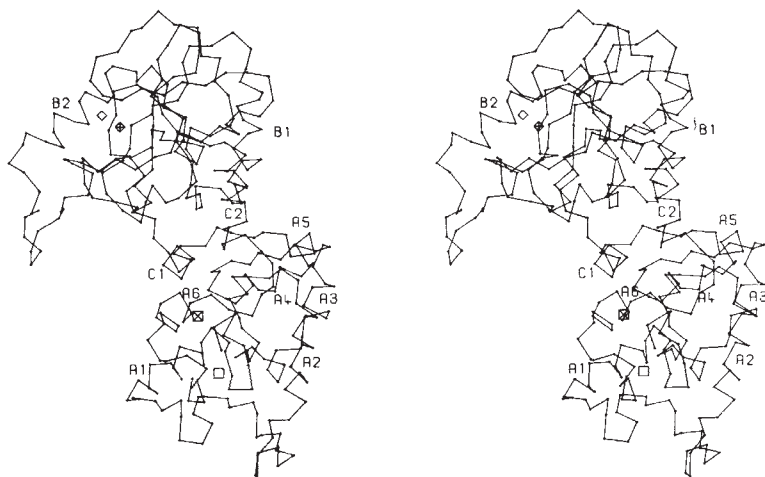
Fig. 1 Sections through the electron density map of PGK (black contours) with the difference electron density map of PGK+(Mn-ADP) minus PGK superimposed. Lowest black contour is at $0.35 \text{ e } \text{\AA}^{-3}$ followed at intervals of $0.15 \text{ e } \text{\AA}^{-3}$. Broken white contours are negative, full ones positive. White contours are drawn at intervals of $0.06 \text{ e } \text{\AA}^{-3}$ with the zero contour omitted. The positions of α carbon atoms are marked with black disks. The axis of helix B2 lies in the plane of the sections and can be seen running from the top centre to middle left hand side of the map. Lobe B is above lobe A as illustrated. The peak assumed to be associated with the metal-phosphate end of the ADP molecule (peak 1) is the peak contoured in white nearest the centre of the map. The difference peak immediately below peak 1 is thought to represent the adenine portion of the ADP molecule. Other difference electron density peaks do not exceed one contour level on any one Fourier section.

in our investigation. We nevertheless believe that the structural information now available may be of considerable use in providing a conceptual framework for those interested in the structure and mechanism of kinases.

The PGK Molecule

The separation of the monomeric PGK molecule into two lobes connected by a central waist region^{1,2} is now confirmed. The polypeptide chain crosses twice between the two lobes, once in each direction. This means that both chain termini must occur in the same lobe, lobe A. The other lobe, lobe B, contains the yeast enzyme's single sulphhydryl group, as judged from the positions of our two heavy atom sites¹.

Fig. 2 Stereoscopic drawing of the PGK molecule viewed in the same orientation as the electron density map shown in Fig. 1. The positions of the α carbon atoms are marked so as to help the viewer follow the course of the main chain. The helical segments are labelled A1–A6, B1–B2, and C1–C2, to indicate the lobe or central waist region and numerical order of occurrence along the chain from the amino end. The positions of the two heavy atom sites are marked by open (Au) and crossed (Hg) diamonds respectively. The positions of the two significant difference Fourier peaks (see text) are shown with open (peak 2) and crossed (peak 1) squares respectively.



Of the total of 357 residues built into our model, 157 are in lobe A and 181 in lobe B, the remaining nineteen residues forming the connecting region between the two lobes.

Each of the two main chain connections between the lobes includes a short helical segment, designated C1 and C2. Inspection of the electron density map also shows five contact areas involving amino acid side chains which could be important in maintaining the two lobes in their relative orientations. On one side of the molecule, a residue near the carboxyterminal end of helix C1 (see stereo diagram) is in contact with a residue in helix A6. The amino terminal residue of helix C1 is in contact with another, non-helix, residue in lobe A, and the preceding residue is in contact with a non-helix residue in lobe B. At the other side of the molecule, two residues in helix C2, separated by a single amino acid, are in contact with residues in lobe A and lobe B respectively.

The molecule as a whole contains ten helices, six in lobe A, two in lobe B, and the two already referred to as forming part of the inter-lobe connection. Altogether some eighty-six residues, or one-quarter of the molecule, are involved in these helical segments, which vary in length from six to thirteen residues.

Although the existence of interchain hydrogen bonds cannot be established directly at this resolution, there are regions in each lobe where sections of extended polypeptide chain run sufficiently close together to suggest that a β pleated sheet is formed. These β pleated sheet regions tend to form the core of each lobe, and seem to be protected from the solvent by the helices, which all lie approximately tangential to, and form part of, the surface of the molecule. In lobe A, three strands running parallel in direction and sense form a plane between helices C2 and A3. Adjacent to this plane, the amino terminal part of the chain folds to form a compact double bend whose three short constituent sections lie close together in a plane between helices A6 and A2. These two possible regions of β pleated sheet also have a short common boundary whose adjacent chains run in opposite directions. In the other lobe, a twisted sheet is formed, between helices B1 and B2, by five chains, the middle one of which runs from the carboxy terminal end of helix B1 (the top of helix B1 as shown in the stereo diagram).

At two places in the molecule, for a distance of one amino acid residue, the main chain seems to pass through negligible electron density. In one further place there is a similar gap at the tip of a loop protruding into the external solvent. Although we have bridged this gap with a single amino acid, the conformation and position of this region would be consistent with the presence of a larger loop. Such a large flexible loop would not be revealed using X-ray diffraction techniques, and might help to explain the discrepancy between the 357 residues built into our model of

the enzyme and the 420 residues calculated to be present using molecular weights determined by other physical methods^{6,7}. We believe, however, that this discrepancy is such as to suggest that solution studies have led to over-estimation of the molecular weight of PGK.

Structure and Reactivity

The heavy metal ligands used in the structure determination bind to the enzyme at two points 6 Å apart between two chains near the surface of lobe B. Although it is not possible at this stage in the investigation to indicate which of these two chains contains the molecule's single cysteine residue, it is quite clear that this residue is some 30 Å from the catalytically active site of the enzyme, as is shown in the stereo diagram. This is consistent with the finding that the cysteine residue is not essential for enzymic activity^{7,8}.

Substrate binding studies with yeast PGK are still at a preliminary stage. Changes in X-ray diffraction intensity have been observed for crystals soaked in the presence of adenosine⁹, and of ADP with and without Mg^{2+} or Mn^{2+} , but not with 3-phosphoglycerate. Complete sets of 5 Å resolution X-ray diffraction data have been collected for the promising derivatives, and various combinations of difference electron density maps have been calculated. These maps show two consistent features, both positive, as is illustrated in Fig. 1, where the peaks associated with the PGK+(Mn-ADP)-PGK difference map are superimposed on equivalent sections of the normal electron density map. These peaks, designated peak 1 and peak 2, are shown in the stereo diagram with crossed and open squares respectively. Both peaks normally seem to be at least three times the general error level and are easily the strongest features in the calculations. In difference electron density maps between PGK+(Mg-ADP or Mn-ADP) and PGK alone, peak 1 is about 50% stronger than peak 2, whilst in the PGK+(Mn-ADP)-PGK+(Mg-ADP) difference map peak 1 is three times as strong as peak 2. These results suggest that peak 1 represents the phosphate-metal end of the ADP molecule whilst peak 2 represents the adenine portion. The two peaks are positioned in space in such a way that an ADP molecule can be built into the observed density which is fully consistent with this interpretation; the nucleotide substrate making very reasonable contacts with the protein without the need for any obvious change in conformation. Presumably the sugar moiety is not seen in the difference maps because it displaces some other molecule or group of molecules when the enzyme, in the crystal, binds ADP.

The picture of the PGK molecule which is now emerging thus focuses our attention on one region in particular. This is the 20 Å-deep wedge-shaped space between the two lobes, with helix C1 at its apex, which is on the same side

of the molecule as the single sulphhydryl group. This area contains, besides several points of inter-lobal contact, the nucleotide binding site. One may therefore expect that the residues involved in the transfer of the bioenergetically important phosphate group will be found in this region. We also note that the main structural features of the nucleotide binding lobe are similar to those which have been shown to surround the NAD binding site of several dehydrogenases¹⁰⁻¹². A detailed comparison of the kinase and dehydrogenase binding sites must await extension of the present investigation. From the evidence now available, however, it seems probable that evolution has produced a general method for binding nucleotide substrates, and that the architecture of this binding site has been conserved for enzymes requiring such a function, including kinases.

Results comparable to those described here for yeast PGK have been obtained by Dr Blake and his colleagues at Oxford for the horse muscle enzyme. We thank the Oxford group for showing us the results of their work before publication. We also thank the Science Research Council for supporting our laboratory and for the award of a research studentship to one of us (T. N. B.).

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- ¹ Wendell, P. L., Bryant, T. N., and Watson, H. C., *Nature new Biol.*, **240**, 134 (1972).
- ² Blake, C. C. F., Evans, P. R., and Scopes, R. K., *Nature new Biol.*, **235**, 195 (1972).
- ³ Dickerson, R. E., Kendrew, J. C., and Strandberg, B. E., *Acta Cryst.*, **14**, 1188 (1961).
- ⁴ Blow, D. M., and Crick, F. H. C., *Acta Cryst.*, **12**, 794 (1959).
- ⁵ Richards, F. M., *J. molec. Biol.*, **37**, 225 (1968).
- ⁶ Larsson-Raźnikiewicz, M., *Eur. J. Biochem.*, **15**, 574 (1970).
- ⁷ Krietsch, W. K. G., and Bücher, T., *Eur. J. Biochem.*, **17**, 568 (1970).
- ⁸ Arvidsson, L., and Larsson-Raźnikiewicz, M., *Biochim. biophys. Acta*, **310**, 430 (1973).
- ⁹ Campbell, J. W., Duée, E., Hodgson, G., Mercer, W. D., Stammers, D. K., Wendell, P. L., Muirhead, H., and Watson, H. C., *Cold Spring Harb. Symp. quant. Biol.*, **36**, 165 (1971).
- ¹⁰ Adams, M. J., Ford, G. C., Koekoek, R., Lentz, P. J., McPherson, A., Rossman, M. G., Smiley, I. E., Schevitz, R. W., and Wonacott, A. J., *Nature*, **227**, 1098 (1970).
- ¹¹ Hill, E., Tsernoglou, D., Webb, L., and Banaszak, L. J., *J. molec. Biol.*, **72**, 577 (1972).
- ¹² Bränden, C. I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I., and Åkeson, Å., *Nature* (1973) submitted.

Production of Superheavy Nuclei by Multiple Capture of Neutrons

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A new calculation of fission barriers and neutron separation energies for heavy neutron-rich nuclei indicates that it is unlikely that superheavy nuclei can be produced either in the astrophysical r process or in man-made nuclear explosions.

PREDICTION of the possible existence of superheavy nuclei has led to attempts to produce them in the laboratory and to searches for them in nature. Calculations of the nuclear properties of such nuclei¹⁻⁴ have predicted half-lives as long as 10^9 yr. Thus, if these nuclei are produced in astrophysical environments, they should be detectable on the Earth and in cosmic rays impinging on the Earth. So far efforts to produce them in the laboratory or to find them in nature have failed.

There has been much interest in whether superheavy nuclei can be produced in the astrophysical r process, which is known to produce many of the naturally occurring neutron-rich nuclei between germanium and bismuth and all of the naturally occurring nuclei heavier than bismuth. The r process, occurring during some catastrophic supernova event, is the capture of neutrons on a time scale much faster than beta decay. Different studies have led to conflicting conclusions⁵⁻¹⁵ as to whether superheavy nuclei can be produced by the r process. The important question is where neutron-induced fission terminates the r-process path in the region of heavy neutron-rich nuclei. This termination depends on how rapidly the effective surface tension of a nucleus decreases with increasing neutron excess and on the extra stability against fission gained

from single particle effects near the neutron closed shell at $N=184$. The treatment of these two effects depends first on an extrapolation of the surface tension of nuclei along the valley of beta stability to nuclei far to the neutron-rich side of the valley of beta stability. Second, it depends on an extrapolation of the additional binding at known closed shells to the predicted closed shell at $N=184$. The various treatments of these two effects have resulted in the conflicting conclusions.

In this paper, we re-examine the question of the formation of superheavy nuclei by the r process, as well as their possible production in thermonuclear explosions, either of the conventional type or of the multistep type proposed by Meldner¹⁶. Figure 1 illustrates the path each of these processes would follow in the neutron-rich region in their approach to the island of superheavy nuclei.

Nuclear Potential Energy of Deformation

Since neutron-induced fission plays the critical role in terminating these processes, we calculate the fission barriers and neutron separation energies for a broad region of heavy neutron-rich nuclei, outlined by the dashed lines in Fig. 1. We use an improved version of the macroscopic-microscopic method, in which the smooth trends of the nuclear potential energy of deformation are calculated from a macroscopic model and the local fluctuations from a microscopic model. This method is described completely in recent review articles¹⁷⁻²¹.

We pay special attention to the effective nuclear surface tension by using the droplet model of Myers and Swiatecki²² for the macroscopic energy. This model takes into account higher order terms in $A^{-1/3}$ and in $[(N-Z)/A]^2$ than are retained in the usual liquid drop model, where A is the mass number and $(N-Z)/A$ is the relative neutron excess. To calculate the microscopic corrections to the energy we use a

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realistic diffuse surface single particle potential^{3,4}. The single particle potential parameters are obtained from statistical calculations²³ and from adjustments to reproduce experimental single-particle levels in heavy deformed nuclei²¹. The approach that we follow here reproduces experimental fission barriers in the actinide region and nuclear ground state masses²¹ to within an accuracy of about 1 MeV and is consistent with information from conventional nuclear explosions in the neutron-rich region²⁴.

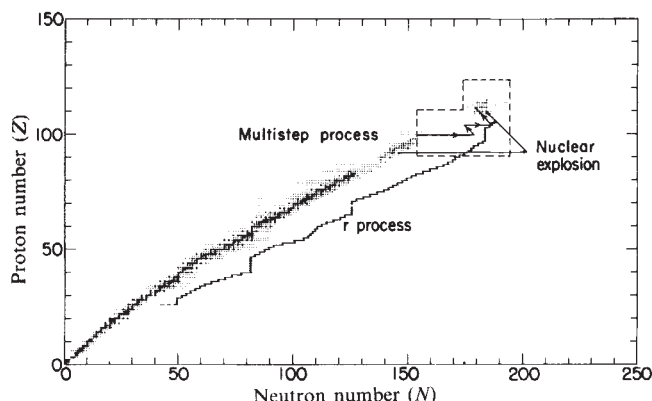


Fig. 1 Plot of known and predicted nuclei as a function of the neutron number N and proton number Z . The heaviest points represent stable nuclei, the medium size points nuclei with half-lives greater than one year, and the lightest points nuclei with half-lives less than one year. Nuclei with predicted half-lives greater than about 5 min are included in the island of superheavy nuclei. The dashed lines outline the region of nuclei where we calculated fission barriers and nuclear ground state masses. Three possible ways to reach the island of superheavy nuclei by the multiple capture of neutrons are illustrated.

The neutron separation energies are calculated from ground state masses that are determined by minimising the potential energy with respect to the nuclear quadrupole moment Q_2 and hexadecapole moment Q_4 . The fission barriers are calculated for a prescribed sequence of nuclear shapes relevant to the fission process. These details are discussed elsewhere.

Effective Surface Tension and Neutron Excess

In the liquid drop model for the macroscopic energy, the surface energy of a spherical nucleus is given by

$$E_s^{(0)} = a_s [1 - \kappa((N-Z)/A)^2] A^{2/3} \quad (1)$$

where a_s is the surface energy constant and κ is the surface asymmetry constant. For a large value of κ , the effective surface tension decreases rapidly with increasing neutron excess, which results in decreased barrier heights for heavy neutron-rich nuclei. In the liquid drop model of Myers and Swiatecki²⁵ the surface asymmetry constant was assumed equal to the volume asymmetry constant, or to the value $\kappa=1.7826$.

In the droplet model the shape dependence of the potential energy is more complicated than in the liquid drop model, and the constant κ no longer enters. But by neglecting the relatively small influence of some of the new shape-dependent energies, the more complicated dependence of the droplet model on the surface energy can be described²⁶ approximately in terms of an effective value of κ . Unlike in the liquid drop model, this effective value of κ depends on the mass number and the nuclear shape.

With the droplet model parameters of Myers and Swiatecki²², we obtain $\kappa_{\text{eff}}=2.8$ for the neutron-rich nucleus ^{280}Pu . Thus

the effective surface tension decreases much more rapidly with neutron excess than in that liquid drop model. A recent determination of the constants of a truncated droplet model that reproduces both experimental ground state masses and fission barrier heights to within an accuracy of 1 MeV yields a value for ^{280}Pu of $\kappa_{\text{eff}} \approx 3.5$ (W. M. H. and P. A. Seeger, in preparation).

Fission-Barrier Heights

Figure 2 is a contour plot of the calculated fission-barrier height for even nuclei. We include a zero-point energy of 0.5 MeV for motion in the fission direction. Notice the large barriers (≈ 10 MeV high) for nuclei in the superheavy region ($N \approx 184$, $Z \approx 114$). The barriers for these superheavy nuclei are similar to those that others calculate with a diffuse surface potential^{3,4,18,20}.

The barrier heights for nuclei in the region $176 \lesssim N \lesssim 192$ are largely the result of single particle effects because the macroscopic energy contribution has almost disappeared due to the large effective surface-asymmetry constant. Notice the pronounced enhancement of barrier heights for nuclei with neutron numbers near $N=184$. In the broad region of nuclei $162 \lesssim N \lesssim 178$, $92 \lesssim Z \lesssim 106$ with relatively low fission barriers the capture of a neutron by these nuclei would lead to disastrous neutron-induced fission.

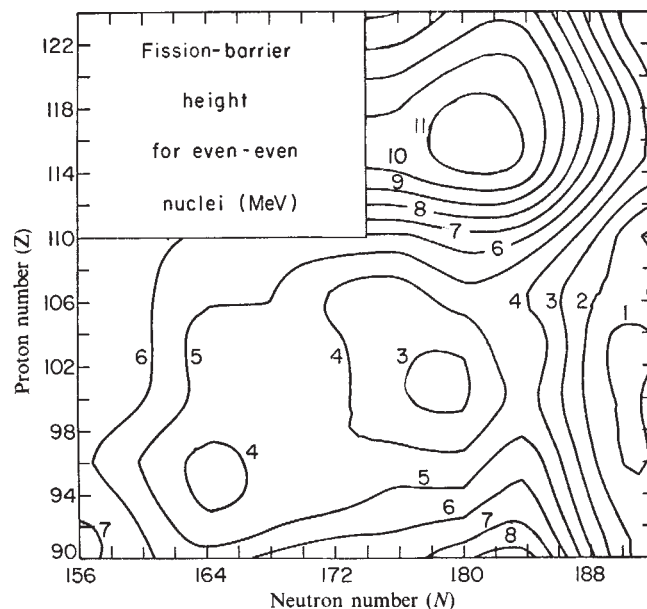


Fig. 2 Contour plot of the calculated fission-barrier height as a function of neutron number N and proton number Z for even nuclei. These fission barriers are calculated with droplet model constants that optimally reproduce nuclear ground state masses and fission barrier heights.

In order to study the sensitivity of the calculated barrier heights to the parameters of the droplet model, we have repeated the fission barrier calculations with two different sets of droplet model constants (W. D. Myers, private communication). These two sets of constants both give a standard deviation between the experimental and calculated ground state masses and fission barrier heights that is 10% larger than the optimum value. The results for these two different sets of droplet model constants are presented in contour plots in Figs 3 and 4. The constants used in the calculation in Fig. 3 yield an effective surface-asymmetry constant for ^{280}Pu of

$\kappa_{\text{eff}}=2.6$, while the constants used in the calculation in Fig. 4 yield $\kappa_{\text{eff}}=3.3$. As expected, the fission barrier heights near the neutron closed shell at $N=184$ are almost independent of the droplet model parameters. The same microscopic corrections have been used for all of the calculations. In our consideration of the sequence of shapes for fission, we have not taken into account mass-asymmetric or axially asymmetric distortions. For some nuclei these effects could lower the calculated barriers by a few MeV.

Neutron Separation Energies

Figure 5 is a contour plot of the calculated neutron separation energy for even nuclei. The neutron separation energies decrease monotonically for increasing neutron excess. For a given determination of the neutron separation energy for

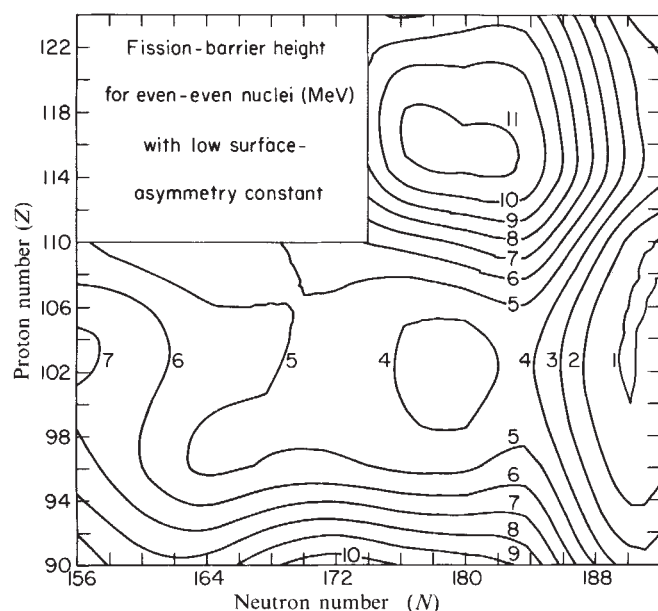


Fig. 3 Analogous to Fig. 2, except calculated with droplet model constants for which the effective surface asymmetry constant is lower. The error in the calculated ground state masses and fission barrier heights is 10% larger with these constants than with those used in Fig. 2.

neutron-rich nuclei, the *r*-process path can be determined approximately without carrying out a full *r* process calculation. Because it is a process in nuclear statistical equilibrium, it follows approximately a line of constant neutron separation energy, whose value we determine to be $B_n=3\pm 1$ MeV for even nuclei.

Termination of Multiple Neutron-capture Processes

Figure 6 presents the essence of our results. When a nucleus captures a thermal neutron, it is excited to an energy equal to its neutron separation energy. When this energy is above the fission barrier height, then the nucleus will undergo neutron-induced fission at a much more rapid rate than the other available decay modes. Figure 6 is a contour plot of the difference between the fission barrier height and the neutron separation energy for even nuclei. From a study of Figs 5 and 6, we find the neutron-induced fission cutoff for the *r* process to be at $Z=96$ and $N=186$. Since the fission barrier heights in this region are almost independent of the macroscopic energy contributions, this cutoff is strongly dependent on the single-particle effects near the closed shell at $N=184$.

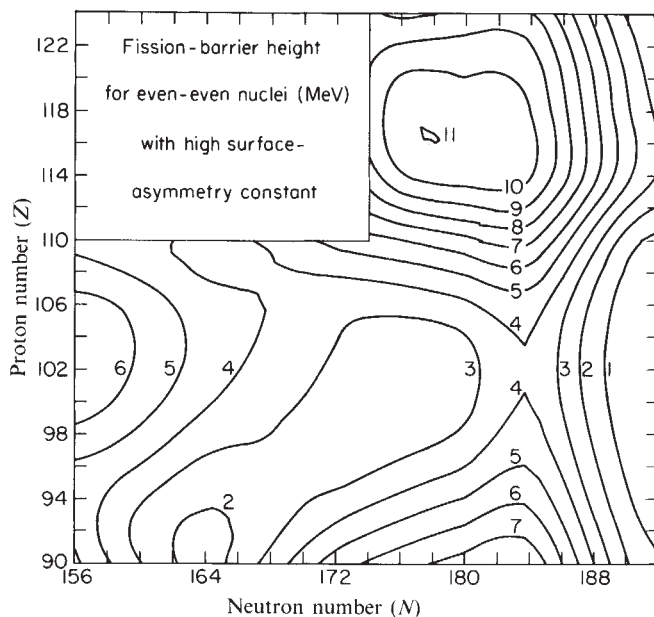


Fig. 4 Analogous to Fig. 2, except calculated with droplet model constants for which the effective surface asymmetry constant is higher. The error in the calculated ground state masses and fission barrier heights is 10% larger with these constants than with those used in Fig. 2.

Thus, we predict that nuclei with mass number $A=281$ will be the last to survive in the *r* process before neutron-induced fission terminates the path. According to the results of ref. 4, nuclei with mass number 281 would spontaneously fission with halflives of the order of 1 s after a few beta decays. In order for superheavy nuclei to be observed in cosmic rays, their halflives would need to be at least 10^6 yr. This would require the production of nuclei with mass number $A \gtrsim 291$, which is 10 mass units higher than the heaviest yield that we predict.

Schramm and Fiset¹³ suggest that a small fraction of the material at $N=184$ could climb along this closed shell during

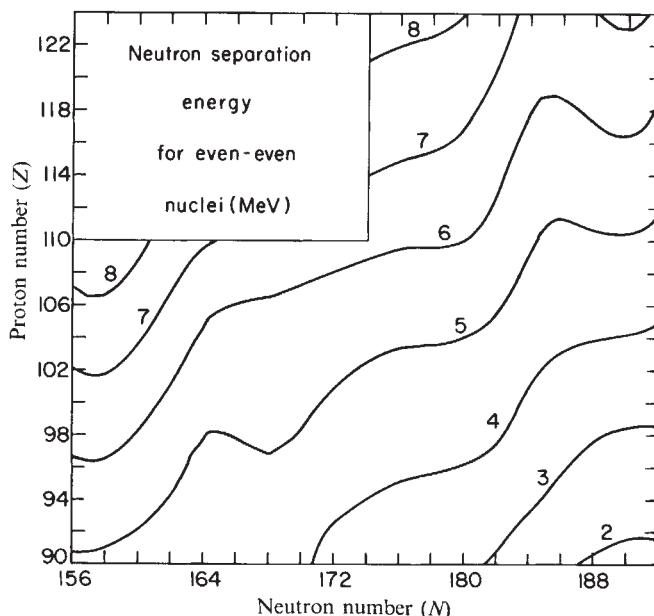


Fig. 5 Contour plot of the calculated neutron separation energy as a function of neutron number N and proton number Z for even nuclei.

a weak freeze out of the *r* process neutron flux and thereby reach the superheavy island. But as seen in Fig. 6, such nuclei would reach the 'cape' close to $Z=104$ and suffer neutron-induced fission before reaching the superheavy island.

Conventional nuclear explosions yield neutron exposures similar to those in the astrophysical *r* process; however, the time duration is so short ($\Delta t \lesssim 10^{-6}$ s) that there is no time for beta decays during the neutron-capture process. The thermal photon temperature is much less than in the *r* process. The neutron captures therefore proceed to a lower neutron separation energy, which extends into a more neutron-rich region. In nuclear explosions to date, targets of various actinide nuclei have been irradiated with intense neutron sources. Independent of the target used or the intensity of the neutron irradiation, the heaviest nucleus recovered from the debris²⁷ has been ^{257}Fm .

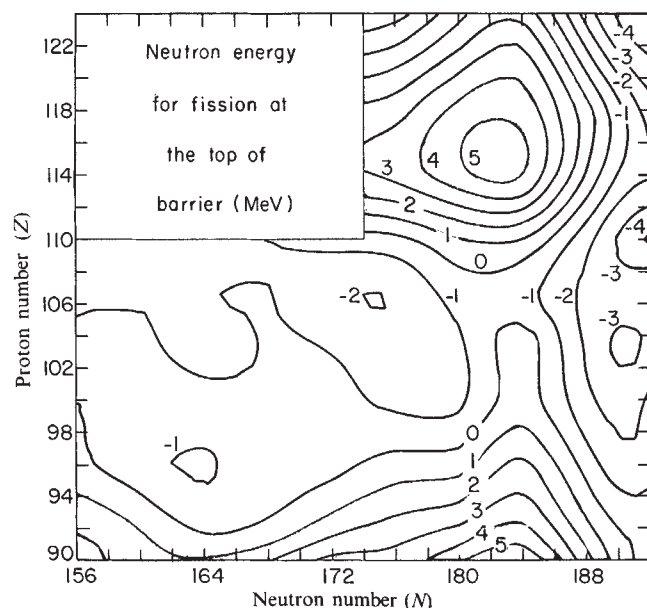


Fig. 6 Contour plot of the difference between the fission barrier height and the neutron separation energy for even nuclei.

We can understand this failure to produce heavier nuclei in terms of our results. From Fig. 6 we see that neutron-induced fission terminates the neutron capture chain on the U isotopes at mass number $A=256$. Actually, neutron-induced fission should occur somewhat before this because we overestimate the barrier for U isotopes in this region by 1 or 2 MeV by not including mass-asymmetric distortions. Thus, we conclude that conventional nuclear explosions have even less hope for producing superheavy nuclei than the *r* process.

The failure of conventional nuclear explosions to produce superheavy nuclei has led Meldner¹⁶ to propose a multistep explosive process that would allow beta decays between subsequent explosions. But his process seems fraught with the same difficulties encountered in the *r* process and in conventional nuclear explosions. Certainly odd-mass capture chains would have to be utilised as well as a target lighter than Th to avoid initial termination of the process by neutron-induced fission. It would then be required that beta decay lead to a region of stability against neutron-induced fission before the next neutron burst.

Meldner would like to take advantage of neutron capture along odd-proton chains where the fission barrier could be enhanced relative to the barrier heights of even nuclei. In

the actinide region the spontaneous-fission half-lives of odd-particle nuclei are systematically about 10^3 times as long as those of neighbouring even nuclei²⁸. These hindrances arise from an increase either in the height of the barrier or in the inertia (or in both). If this hindrance factor is assumed to arise only from an increase in the barrier height, then the barriers for odd-particle nuclei are raised by about 0.5 MeV relative to those for even nuclei¹.

An examination of Fig. 6 reveals that after an initial neutron burst and subsequent beta decay into $162 \lesssim N \lesssim 178$, $92 \lesssim Z \lesssim 106$, the nuclei would be in a region where a second neutron burst would initiate neutron-induced fission. This conclusion also applies for odd-proton capture chains. But as we have seen, the relatively strong dependence of the fission-barrier heights in the region of $162 \lesssim N \lesssim 178$, $92 \lesssim Z \lesssim 106$ on the value of the effective surface-asymmetry constant makes this conclusion somewhat uncertain.

To summarise, we have used an improved version of the macroscopic-microscopic method to calculate fission barriers and neutron separation energies for a broad region of heavy neutron-rich nuclei. On the basis of these calculations we conclude that neutron-induced fission terminates the three possible multiple-neutron-capture processes well before the superheavy island is reached.

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- ¹ Myers, W. D., and Swiatecki, W. J., *Nucl. Phys.*, **81**, 1 (1966).
- ² Nilsson, S. G., Tsang, C. F., Sobczewski, A., Szymański, Z., Wycech, S., Gustafsson, C., Lamm, I. L., Möller, P., and Nilsson, B., *Nucl. Phys.*, **A131**, 1 (1969).
- ³ Bolsterli, M., Fiset, E. O., Nix, J. R., and Norton, J. L., *Phys. Rev. C*, **5**, 1050 (1972).
- ⁴ Fiset, E. O., and Nix, J. R., *Nucl. Phys.*, **A193**, 647 (1972).
- ⁵ Seeger, P. A., Fowler, W. A., and Clayton, D. D., *Astrophys. J. Suppl.*, No. 97, **11**, 121 (1965).
- ⁶ Viola, jun., V. E., *Nucl. Phys.*, **A139**, 188 (1969).
- ⁷ Berlovich, E. E., and Novikov, Yu. N., *JETP Lett.*, **9**, 445 (1969).
- ⁸ Cowan, G. A., in *Proc. Robert A. Welch Foundation Conferences on Chemical Research, XIII, The Transuranium Elements—The Mendeleev Centennial*, 291 (Welch Foundation, Houston, 1970).
- ⁹ Schramm, D. N., and Fowler, W. A., *Nature*, **231**, 103 (1971).
- ¹⁰ Ohnishi, T., *Progr. theor. Phys.*, **47**, 845 (1972).
- ¹¹ Boleu, R., Nilsson, S. G., Sheline, R. K., and Takahashi, K., *Phys. Lett.*, **40B**, 517 (1972).
- ¹² Boleu, R., *Nucl. Phys.*, **A201**, 401 (1973).
- ¹³ Schramm, D. N., and Fiset, E. O., *Astrophys. J.*, **180**, 551 (1973).
- ¹⁴ Brueckner, K. A., Chirico, J. H., Jorna, S., Meldner, H. W., Schramm, D. N., and Seeger, P. A., *Phys. Rev. C*, **7**, 2123 (1973).
- ¹⁵ Johns, O., and Reeves, H., *Astrophys. J. Lett.* (in the press).
- ¹⁶ Meldner, H. W., *Phys. Rev. Lett.*, **28**, 975 (1972).
- ¹⁷ Johansson, T., Nilsson, S. G., and Szymański, Z., *Ann. Phys., Paris*, **5**, 377 (1970).
- ¹⁸ Brack, M., Damgaard, J., Jensen, A. S., Pauli, H. C., Strutinsky, V. M., and Wong, C. Y., *Rev. mod. Phys.*, **44**, 320 (1972).
- ¹⁹ Nix, J. R., *A. Rev. nucl. Sci.*, **22**, 65 (1972).
- ²⁰ Pauli, H. C., *Phys. Rep.*, **7**, 35 (1973).
- ²¹ Möller, P., and Nix, J. R., Paper IAEA-SM-174/202, in *Proc. third IAEA Symp. Physics and Chemistry of Fission* (to be published).
- ²² Myers, W. D., and Swiatecki, W. J., *Berkeley Preprint*, LBL-1957 (1973).
- ²³ Myers, W. D., *Nucl. Phys.*, **A145**, 387 (1970).
- ²⁴ Howard, W. M., and Nix, J. R., Paper IAEA-SM-174/60, in *Proc. third IAEA Symp. Physics and Chemistry of Fission* (to be published).
- ²⁵ Myers, W. D., and Swiatecki, W. J., *Ark. Fys.*, **36**, 343 (1967).
- ²⁶ Seeger, P. A., in *Proc. fourth int. Conf. Atomic Masses and Fundamental Constants*, 255 (Plenum, London, 1972).
- ²⁷ Eccles, S. F., in *Proc. Symp. Engineering with Nuclear Explosives*, CONF-700101, **2**, 1269 (USAE Division of Technical Information, Springfield, 1970).
- ²⁸ Hyde, E. K., *The Nuclear Properties of the Heavy Elements, Fission Phenomena*, **3**, 50 (Prentice-Hall, Englewood Cliffs, 1964).

LETTERS TO NATURE

PHYSICAL SCIENCES

Model for Cygnus X-3

CYGNUS X-3 has been identified¹ with the infrared object found by Becklin *et al.*² to be within $\pm 2''$ of the radio position: the infrared object is found to vary in intensity with the same period as and in phase with the X-ray object. The infrared flux (2.2 μm) varies smoothly by about 15% whereas the X-ray intensity varies by a factor ~ 3 . Cyg X-3 is unique among other regularly varying sources in that the X-ray light curve is smooth and roughly sinusoidal³, displaying no sudden intensity variations indicative of an eclipse of a compact X-ray source by a larger object (for example, a companion star). We show that nevertheless Cyg X-3 can be interpreted by means of the 'standard model' for galactic X-ray sources in terms of mass loss from a large companion star and accretion onto a compact object.

We assume throughout that the 4.8 h period is due to orbital motion. Kepler's Law (assuming a circular orbit) yields

$$D \approx 10^{11} (M/M_{\odot})^{\frac{1}{3}} \text{ cm} \quad (1)$$

where D is the distance between the two stars and M is the total mass of the system. If this is also the size of the infrared object (or an upper limit to it) we can obtain a lower limit to the brightness temperature at 2.2 μm (see ref. 1)

$$T_B \gtrsim 3 \times 10^6 (M/M_{\odot})^{-\frac{2}{3}} (d/10 \text{ kpc})^{\frac{1}{2}} \text{ K} \quad (2)$$

where d is the distance to the source. From radio data⁶ we know that $d \gtrsim 10 \text{ kpc}$.

If the bulk of the infrared flux is due to radiation from the surface of an ordinary star we need⁴ $M \gtrsim 70 M_{\odot}$. In this case the 2.2 μm variation cannot be due to tidal effects—this would give two infrared minima per X-ray period—and the most likely explanation would be that it is a reflection effect. For a 15% variation in the infrared, the X-ray source must give a variation in the energy flux over the stellar surface of nearly a factor of two. This is possible, but, if so, the reflection effect could easily give rise to all the infrared emission, obviating the need for such a massive system. I shall therefore assume the standard model: X rays originate from a compact object (I show below how the light curve may be produced) and the infrared flux is due to heating of the companion star (or its extensive atmosphere or wind) by the X-ray star.

The observed mean X-ray luminosity in the 2 to 6 keV range is⁵

$$L_X \approx 3 \times 10^{37} (d/10 \text{ kpc})^2 \text{ erg s}^{-1} \quad (3)$$

If the infrared emission comes from the Rayleigh-Jeans tail of radiation from an optically thin source emitted by a gas at temperature T_B , the total luminosity of the gas is given by

$$L_g \gtrsim 4 \times 10^2 L_{\text{IR}} (M/M_{\odot})^{-\frac{2}{3}} \quad (4)$$

where L_{IR} is the luminosity at 2.2 μm . Using the dereddened flux at 2.2 μm (ref. 1)

$$L_X \approx 4 \times 10 L_{\text{IR}} \quad (5)$$

Thus the total X-ray luminosity must be given by $L_{\text{XT}} = f L_X$ where

$$f \gtrsim 10(4\pi/\Omega)(1-\alpha)^{-1}(M/M_{\odot})^{-\frac{2}{3}} \quad (6)$$

Ω is the solid angle subtended by the gas from the X-ray source and α is the albedo. Thus to power the infrared source we require a total X-ray luminosity in excess of that observed in the 2 to 6 keV range. That is, the bulk of the X-ray luminosity is emitted either above 6 keV or below the low energy cut-off at about 3 keV. This is similar to what is required in the system Her X-1/HZ Her (ref. 7 and Y. Avni *et al.*, to be published). If the inclination of the orbit i is moderate (say $30^\circ \lesssim i \lesssim 60^\circ$), variation in aspect of the gas and of the amount of gas visible can easily produce the required smooth 15% variation.

I now outline a possible model for producing the X-ray light curve. I suggest that the smooth variation of the X-ray luminosity is produced by a gradual and partial eclipse of the (compact) X-ray object by a stellar wind emanating from its larger companion. This same wind can also give rise to the mass transfer required to power the X-ray source. The 2 to 6 keV spectrum has a variable low energy cut-off, but this variation does not appear to be correlated with the 4.8 h period (P. Sanford, private communication). Thus, and also for simplicity, I assume that the main opacity in the wind is electron scattering. For moderate inclination i we shall see that the electron scattering optical depth τ_{es} can vary by order unity over the orbital period. Thus if $\tau_{\text{es}} \sim 1$, the luminosity of the compact source will seem to vary by ~ 3 . If, however, absorption is unimportant everywhere in the wind, backscattering of X rays would provide a diffuse background X-ray luminosity comparable to that of the compact object. So to obtain the required X-ray variation, we need absorption to be important but not dominant.

For the sake of argument I limit discussion to a specific model of the wind. I assume (see ref. 7) that the wind is emitted by the star in a spherically symmetric fashion at about the escape velocity, and that the parts of the wind where absorption and scattering take place are dynamically unaffected by the presence of the X-ray source. Conservation of mass implies that the wind density falls off as the inverse square of the distance from the star. The electron scattering optical depth is given as a function of θ , the angle between the line of sight and the axis of symmetry of the system by

$$\tau_{\text{es}} = 0.2 \times 10^{-24} N D g(\theta) \quad (7)$$

where

$$g(\theta) = (\pi - \theta)/\sin \theta \quad (8)$$

and N is the number density at distance D from the large star. The unscattered X-ray flux seen at infinity, L_U , is given in terms of the source luminosity L_S by

$$L_U = L_S \exp \{ -\tau_{\text{es}}(\theta) \} \quad (9)$$

and the X-ray light curves obtained using this are shown in Fig. 1 for various values of the inclination. For each value of i , $\tau_{\text{es}}(\pi/2)$ has been chosen such that the overall luminosity variation is by about a factor of three. Note the similarity between these curves and the actual light curve of Cyg X-3.

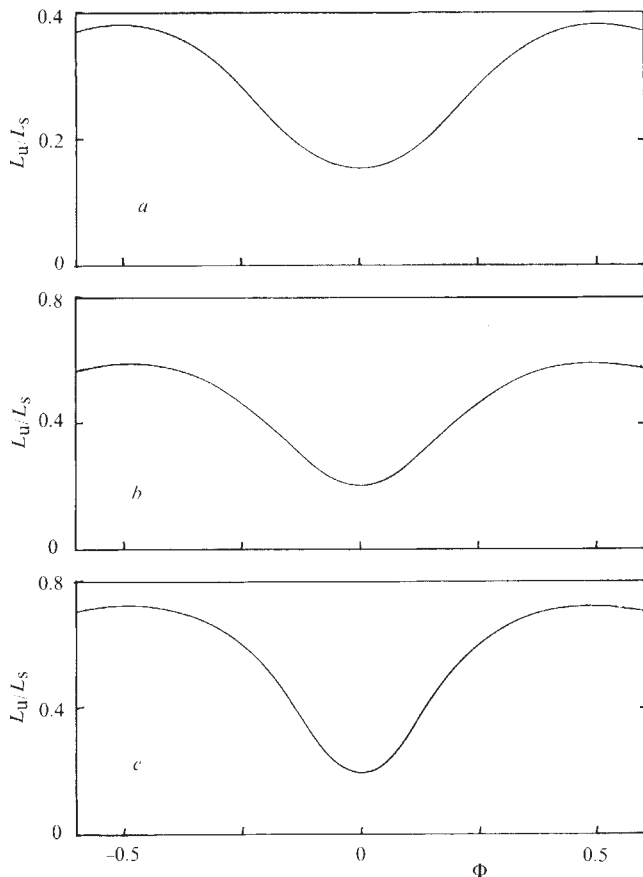


Fig. 1 The fraction of X-ray flux that reaches the observer unscattered, L_u/L_s , is plotted against binary phase Φ for various values of the inclination i and the optical depth to electron scattering $\tau_{es}(\pi/2)$. *a*, $i=30^\circ$, $\tau_{es}(\pi/2)=1.25$; *b*, $i=45^\circ$, $\tau_{es}(\pi/2)=0.75$; *c*, $i=60^\circ$, $\tau_{es}(\pi/2)=0.5$. For each i , the value of $\tau_{es}(\pi/2)$ has been chosen to give an overall variation of about a factor of three.

I argued earlier that absorption in the wind must be important but not dominant. That is, we must have

$$\lambda = F/(4\pi D^3 N^2 a) \simeq 1 \quad (10)$$

where F is the ionising photon flux and a the effective recombination coefficient (see ref. 7 for a fuller discussion). Using $a \sim 3 \times 10^{-10} T^{-3/2}$, we obtain

$$N \approx 10^{13} (M/M_\odot)^{-1/2} L_{38}^{1/2} T_6^{3/2} \text{ cm}^{-3} \quad (11)$$

where L_{38} is the X-ray luminosity in units of $10^{38} \text{ erg s}^{-1}$, T_6 is the temperature of the wind in units of 10^6 K and I have assumed the typical ionising photon to have an energy of 5 keV. This density implies

$$\tau_{es}(\pi/2) \approx 0.5 (M/M_\odot)^{-1/2} L_{38}^{1/2} T_6^{3/2} \quad (12)$$

in rough agreement with previous estimates. This density also indicates that the overall mass loss rate from the large star must be

$$\dot{M}_1 \approx 10^{-6} (M/M_\odot)^{1/2} (M_1/M) L_{38}^{1/2} T_6^{3/2} M_\odot \text{ yr}^{-1} \quad (13)$$

where M_1 is the mass of the large star. So for this model we expect the 4.8 h period to change on a time scale of $\sim 10^6 \text{ yr}$.

I note also that inhomogeneities in the wind and variations of the wind strength can lead to irregular variations in the low energy cut-off. Absorption is more important near X-ray minimum, and so the X-ray curves calculated on the basis of electron scattering alone will be distorted downwards near minimum. Thus the variation will be less pronounced at high energies than at low energies where absorption is more impor-

tant. I note in passing that a mass loss rate of $\sim 10^{-3} M_\odot \text{ yr}^{-1}$ of the kind suggested by van den Heuvel and De Loore for the source Cen X-3 (ref. 8) would entirely extinguish any X-ray flux from the compact object. Unless the velocity of the wind exceeds the escape velocity from the system by several orders of magnitude, the wind density required would imply $\tau_{es}(\pi/2) \gg 1$ and $\lambda \ll 1$.

This model predicts that a large fraction of the total X-ray luminosity is absorbed in the stellar wind. Basko and Sunyaev⁹ show that for stars with a high mass loss rate most of the energy is absorbed by gas with $T \sim 10^6 \text{ K}$ (comparable to T_b). Most of this energy is re-radiated as ultraviolet and as soft X rays from a region whose size is comparable to that of the system. This confirms that the spectrum from the gas will be flat out to wavelengths as long as $2.2 \mu\text{m}$, before turning over to Rayleigh-Jeans at longer wavelengths. If this soft X-ray flux were visible we expect it to vary in a similar way to the infrared flux. The hard X-ray flux varies by a much greater amount and so cannot come from the same region. Also, the optical flux predicted here is much less than that expected from a straightforward extrapolation of the infrared flux along the Rayleigh-Jeans curve, as would be the case if the bulk of the infrared flux came from the stellar surface. Finally, I stress again the uniqueness of the system Cyg X-3 and, in a sense, the uniqueness of this model. For the parameters of Cyg X-3 we can have $\tau_{es} \sim 1$ and $\lambda \sim 1$. If the system were larger or smaller we could not satisfy both of these for any wind density, since $\lambda \tau_{es}^2 \propto D^{-1}$, independent of N .

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- ¹ Becklin, E. E., Hawkins, F. J., Mason, K. O., Matthews, K., Neugebauer, G., Sanford, P. W., and Wynn-Williams, C. G., *Nature phys. Sci.*, **245**, 302 (1973).
- ² Becklin, E. E., Kristian, J., Neugebauer, G., and Wynn-Williams, C. G., *Nature phys. Sci.*, **239**, 130 (1972).
- ³ Sanford, P. W., and Hawkins, F. H., *Nature phys. Sci.*, **239**, 135 (1972).
- ⁴ Bruhweiler, F., *Nature phys. Sci.*, **244**, 68 (1973).
- ⁵ Canizares, C. R., McClintock, J. E., Clark, G. W., Lewin, W. H. G., Schnopper, H. W., and Sprott, G. F., *Nature phys. Sci.*, **241**, 28 (1973).
- ⁶ Laque, R., Lequeux, J., and Nguyen-Quant-Rieu, *Nature phys. Sci.*, **239**, 120 (1972).
- ⁷ Pringle, J. E., *Nature phys. Sci.*, **243**, 90 (1973).
- ⁸ Van den Heuvel, E. P. J., and De Loore, C., *Nature phys. Sci.*, **245**, 117 (1973).
- ⁹ Basko, M. M., and Sunyaev, R. A., *Astrophys. Space Sci.*, **23**, 117 (1973).

Close Pairs of QSOs

It has recently been argued^{1,2} that the observation of close pairs of QSOs with very different redshifts provides support for non-cosmological explanations of the redshifts. We show in this note that the pairs observed so far may be comfortably explained as random coincidences and that the small probabilities that are often quoted depend on the use of *a posteriori* statistics.

The expected number of close pairs of QSOs is given by

$$\mathcal{N} = N_Q (\pi R^2) \rho(\leq m) \quad (1)$$

where N_Q is the total number of QSOs whose redshifts have

been determined, R (in arc s) is the 'region of interest' around any given QSO (or the maximum allowable separation for 'close' pairs), and $\rho(\leq m)$ is the total number of QSOs per square arc s down to a limiting magnitude m . Equation (1) may be rewritten inserting typical numbers³, as

$$\mathcal{N}(R, m) \approx 7 \times 10^{-2} (N_0/1,000) (R/10'')^2 10^{0.6(m_v-19)} \quad (1')$$

It should be noted that the typical *a posteriori* probabilities, calculated with the aid of equation (1), for the chance occurrence of a pair of close QSOs are not terribly small; they roughly correspond to 3 σ events.

We recall the basic observational facts. There are now two known 'close' pairs of QSOs: Ton155, 156 (ref. 1) with a separation of 35", redshifts $z=1.7$ and 0.55, and $m_v \approx 16.0$ and 16.6 mag; and 1548+115 a, b (ref. 2) with a separation of 5", redshifts of $z=0.44$ and 1.9, and $m_v \approx 17$ and 19 mag.

We note that the initial observation¹ of a close pair of QSOs, Ton155 and Ton156, could not be regarded by itself as statistically significant because the typical correlation-separation previously discussed⁴ was several degrees (yielding for typical values of R and m in equation (1) values of $\mathcal{N} \gg 1$). But we might legitimately have used the initial observation to define an hypothesis to be tested, for example: QSOs brighter than $m_v=17$ mag have a much higher chance of appearing close together, $R \leq 35''$, than would be expected from a random distribution. On the basis of this hypothesis, the subsequent observation of the close pair 4C11.50 a, b (= 1548 and 115 a, b) is not remarkable. First, m_v (1548 and 115 b) is ~ 19 mag, much fainter than either of the Ton objects, so that the above defined hypothesis does not even cover this case. If we extend the limiting magnitude down to 19.4 mag (so it comfortably covers the observed pair 4C11.50 a, b), then $P(35'', 19.4) \sim 0.5$ compared with the observed value of 2. Actually even this calculation underestimates the expected number of pairs on the cosmological hypothesis. There is no *a priori* reason to suppose that $m=19$ mag or even $m=19.4$ mag is especially significant and one will always get unrealistically small probabilities for events if one calculates after the fact the chance expectation using the already observed parameters to define the experiment. In fact, experience shows that *a posteriori* probabilities at the 1% level are not particularly uncommon for random events; but of course *a posteriori* probabilities at the 10^{-6} or 10^{-10} level still are.

It is interesting to estimate the number of close QSO pairs that one would expect *a priori* on the cosmological hypothesis down to the limiting magnitude (~ 21 mag) of the blue Sky Survey (on which most of the identifications are made). For a total of 10^{-3} QSOs with known redshifts (perhaps a reasonable expectation for the next several years) and a maximum separation of 10", we expect one or possibly a few pairs. If we enlarge the maximum separation to 30" we expect about ten close pairs. These estimates are of course uncertain by at least a factor of three because we do not yet have an accurate determination of the QSO spatial density at faint magnitudes. Nevertheless, close pairs of QSOs should be rather common when it becomes standard practice to measure redshifts for all faint stars near accurate radio source positions.

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¹ Stockton, A. N., *Nature phys. Sci.*, **238**, 37 (1972).

² Wampler, E. J., Baldwin, J. A., Burke, W. L., Robinson, L. B., and Hazard, C., *Nature*, **246**, 203 (1973).

³ Setti, G., and Woltjer, L., *Proc. sixth Texas Symp.* (in the press).

⁴ Arp, H. C., in *The Redshift Controversy* (edit. by Field, G., Arp, H., and Bahcall, J.) (Addison-Wesley, New York, 1973).

Response of Dayside Thermosphere to an Intense Geomagnetic Storm

INCREASES in the density of the neutral thermosphere associated with geomagnetic activity were first noted by Jacchia¹. Although the thermosphere will not be in equilibrium, and acceleration by electrodynamic forces may be involved, on balance the increased density must reflect additional heating. Further studies²⁻⁴ by orbital drag analysis from observations of artificial Earth satellites have led to a complex and somewhat confusing picture. The heating seems to be slightly greater^{3,4} at high latitudes and, for larger disturbances, sometimes much greater^{2,3}. Also, the heating seems to be slightly greater^{3,4} on the nightside, but possibly with the opposite trend³ for larger disturbances ($K_p > 5$). Jacchia *et al.*³ also found that the time delay between the peak of the storm, as measured by the planetary 3-h indices K_p or a_p , and the maximum response was a little shorter at high latitudes; on the other hand Roemer⁴ concluded that the time delay was independent of latitude, height or local time.

The low g accelerometer calibration system (LOGACS) experiment^{5,6} carried on an attitude-controlled Agena vehicle launched by the United States Air Force on May 22, 1967, provided a unique opportunity to determine the response to an intense storm. As the histogram in Fig. 1 shows, the planetary 3-h geomagnetic index, a_p , started to increase on May 25, reached a peak of 236, then fell slightly before increasing to the conventional maximum of 400 for two successive 3-h periods. Figure 1 also gives the hourly equatorial Dst index⁷ which is based only on the horizontal H components at low latitude observatories and effectively measures the strength of the ring current during the storm.

The LOGACS accelerometer provided values of atmospheric density with an estimated accuracy better than 10% and a time resolution of 1 s, although at much longer intervals. Using these values, DeVries^{5,6} found that the density in the (northern) nightside auroral regions increased with the onset of geomagnetic activity whereas the density in the (northern) dayside auroral regions initially decreased. The density over the rest of the Earth apparently reached a maximum with a time delay ranging from near zero in the nightside auroral regions to about 6 h at the dayside equator. DeVries concluded that most of the energy was injected in the nightside auroral regions probably, he suggested, by Joule heating⁸ at or below 150 km, and transported over the rest of the Earth by convective circulation and atmospheric gravity waves.

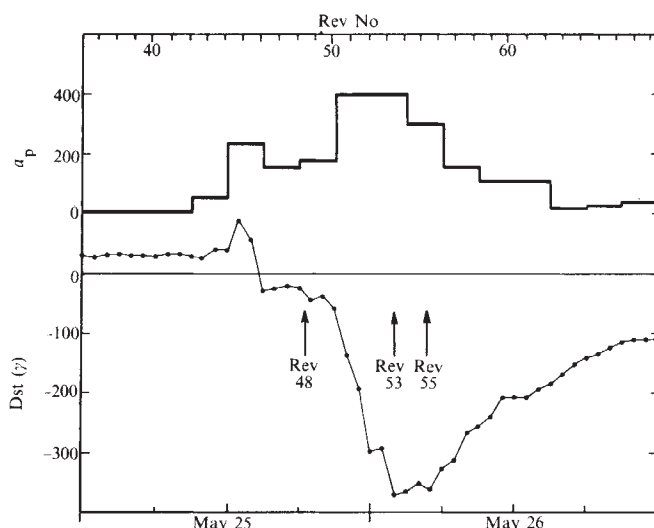


Fig. 1 Geomagnetic activity during the storm as given by the planetary 3 h index a_p (histogram) and the hourly equatorial Dst index. Rev, revolution.

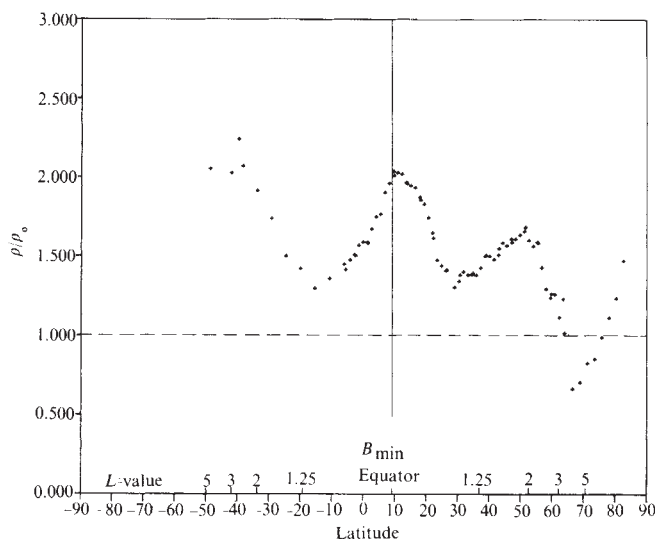


Fig. 2 The density ratio p/p_0 , where p_0 is a standard quiet-time density, for the dayside pass on revolution 53 in the LOGACS experiment. The height of the satellite, which is near polar and passes southwards down the dayside at 1030 LT, is 165 km at 80° N falling to a minimum of 140 km at 45° N, then rising to 165 km at the equator and 305 km at 80° S. The magnetic equator and the feet of various L shells are also shown.

At the invitation of DeVries, we have made an independent analysis (R. R. A. and G. E. Cooke, unpublished) of the LOGACS data in which we concentrated on the dayside passes as these are more complete and contain the most clearly defined structures. The orbit is near-polar with the north-going passes on the nightside at about 2230 LT, and the south-going passes on the dayside at about 1030 LT. Perigee, at a height of 140 km, occurred on the dayside near 45° N, and the apogee height was between 300 and 400 km. For the dayside passes the response in both hemispheres can be compared, whereas on the nightside the vehicle is above 300 km, where the density values are much sparser and less reliable, south of the equator. The most clearly developed dayside response appears on revolution 53 just as the Dst index reaches its negative peak. The response is shown in Fig. 2 in the form of the ratio of the density on revolution 53 to a standard quiet-time density p_0 , which has been derived (R. R. A. and G. E. Cooke, unpublished) from all the quiet-time passes up to revolution 41. Unfortunately, there is a gap in the latitude coverage from 50° S to 80° S in Fig. 2, but revolution 55 is essentially complete and is shown in Fig. 3.

The two figures are very similar; excepting the gap in Fig. 2, they show a wave-like pattern with a central maximum at 10° N, further maxima at 50° N and 40° S, and yet further maxima approaching the polar caps. The pattern is more nearly symmetrical (in form if not in magnitude) in magnetic coordinates, with the central peak on the magnetic equator and the mid-latitude peaks near $L=2$ in the Northern Hemisphere but perhaps nearer $L=3$ in the Southern Hemisphere. This slight asymmetry could result from the greater height in the Southern Hemisphere; alternatively the geometry could be imposing a compromise between geographic and geomagnetic latitudes. On the other hand, the greater magnitude of p/p_0 in the Southern Hemisphere probably arises simply from the greater height.

Although the gross structure of the response has a wave-like pattern, it is not a propagating wave. The dayside pass on revolution 54 is incomplete, but what there is shows the same latitude dependence and densities intermediate between revolution 53 and revolution 55. There are certainly small-scale wave-like features, such as the double peak at 50° N in Fig. 3, but I hesitate to interpret them as evidence for atmospheric waves.

The appearance of pronounced troughs near $\pm 60^\circ$ magnetic latitude is in complete agreement with DeVries^{5,6}. The northern trough appears in both figures and reveals a minimum density of only 70% of the quiet-time value (at a height of 150 km) on revolution 53, rising to 85% by revolution 55. Two possible reasons might be that the air has been accelerated and displaced by electrodynamic forces or that the air is colder, due to the displacement of part of its normal heat source to lower latitudes.

The response time of the dayside thermosphere must be fairly short because the density at the peaks is at, or nearly at, its maximum on revolution 53. At the mid and high-latitude peaks the density perhaps increases marginally by revolution 55. The equatorial peak, however, has nearly disappeared by revolution 55, possibly because ion drag can offer negligible resistance to an expansion in latitude. On the other hand, the density between the peaks continues to increase for some hours. Eventually the structure collapses in irregular fashion—so irregularly that it might be said to disintegrate. The rapidity of the response and its approximate symmetry in magnetic coordinates indicate that the structure shows simply where the energy is injected into the atmosphere, at least in the morning sector, in an intense storm, namely, near the magnetic equator, near the particle precipitation zones which are displaced equatorwards in the storm and over the polar caps.

Moreover, the peak values of p/p_0 at the greater heights in the Southern Hemisphere exceed the corresponding peaks in the Northern Hemisphere and seem to be attained just as rapidly. The implication seems to be that a significant part of the energy input must raise the temperature fairly uniformly throughout the height range and is therefore possibly due to particle precipitation rather than Joule heating.

The most interesting feature of the response is the pronounced peak on the magnetic equator on revolution 53. As this pass coincides with the maximum negative excursion (-370γ) of the Dst index, the obvious conclusion is that the equatorial peak is related to the growth and decay of the storm-time ring current. This conclusion is reinforced (R. R. A., unpublished) by the appearance of a similar, if less marked, peak on revolution 48 soon after the first negative excursion of Dst (Fig. 1). As has been pointed out⁹⁻¹¹, an important loss process for ring current protons will be the charge-exchange collision with neutral hydrogen atoms. The collision results in a fast neutral hydrogen atom which is later stripped at low altitudes (if it goes downwards) to recover a proton, which is then trapped until a third atmospheric interaction removes it, and so on. Subsequently other loss mechanisms, pitch-angle

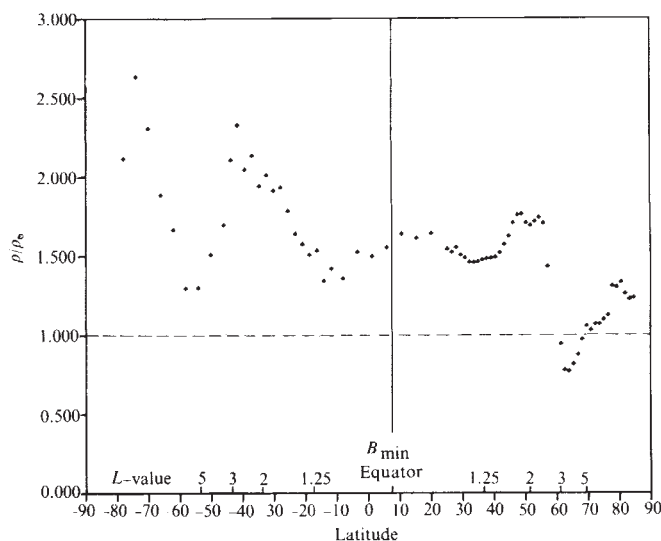


Fig. 3 The corresponding density ratio for the dayside pass on revolution 55, with the details as for Fig. 2.

diffusion due to Coulomb scattering or plasma instabilities, have been extensively discussed and the charge-exchange collision has been somewhat overlooked. Recently, however, new calculations¹² using data on hydrogen densities in the geocorona show that the charge-exchange collision ought to be the dominant loss mechanism below $L=4$. For a large storm, the ring current energy will typically¹³⁻¹⁵ peak somewhat above $L=3$ with a sharp cutoff below $L=3$.

This interpretation of the equatorial peak is confirmed by recent particle measurements below $L=1.15$, which is the lower limit for orbits which can drift completely around the Earth. Moritz¹⁶ finds an ever-present flux of protons near the magnetic equator which he interprets as resulting from charge-exchange losses from the quiet-time ring current. The flux is constant at quiet times (which is consistent with both production and loss processes being proportional to atmospheric density) but increases by up to a factor of three during geomagnetic activity; in fact the energy range (250 keV to 1.65 MeV) of the detector is well above the energy (5 to 50 keV) typical^{13,14} of storm-time ring current protons. More extensive results¹⁷ are now available from OV1-17 for the energy range 12.4 to 500 keV at about 400 km near the magnetic equator at local midnight. The flux of protons below 100 keV increases sharply at disturbed times, but above 200 keV the flux is relatively stable and in agreement with the AZUR results¹⁶. Mizera and Blake¹⁷ find that the flux near 40 keV is particularly well correlated with the growth of the ring current.

If the interpretation is correct, the existence of a response sharply peaked about the equator allows one interesting deduction. At this height (~ 160 km) the lifetimes of any charged particles produced by the precipitating neutral hydrogen atoms must be much shorter than their bounce periods so that the response must reflect the energy input. For a dipole field line $r=L\cos^2\Lambda$, the centre of curvature at the equator lies at $2L/3$. Thus, the narrowness of the response cannot result from a geometrical focusing effect unless the source lies below about $L=2$, which seems unlikely, or unless the field is grossly distorted by the currents flowing. It therefore seems more probable that the part of the storm-time ring current responsible for the equatorial peak must be confined fairly closely to the equatorial plane. In support of this view, Frank¹⁴ has noted that his observations "strongly suggest that these protons are injected into the outer radiation zone as an 'equatorial plasma jet' during the initial phase of a magnetic storm". Similarly, Sugiura¹⁸ finds that the quiet-time ring current is closely confined to the equatorial plane.

The near disappearance of the equatorial peak by revolution 55 also calls for further comment. One possible reason would be that the storm-time ring current particles have acquired significant bounce amplitudes, so that the energy is being deposited over a wider latitude band. It seems more likely, however, that the closest part of the ring current has been removed by revolution 55, in line with the estimated¹² charge-exchange lifetime of 3 to 10 h at $L=3$. For a storm of magnitude -150γ on April 17, 1965, Cahill¹³ finds that a large part of the energy between $L=2.5$ and $L=3.5$ (estimated to be $\sim 10^{21}$ erg) is lost in less than 2 h.

The interpretation of the equatorial response as resulting from charge-exchange losses from the ring current is also in agreement with the results of May and Miller¹⁹ on the response of the equatorial atmosphere (at about 310 km near noon local time) from the decay of the spin rate of Ariel 2 in March and April 1964. They noted that the density variations were more closely correlated with Dst than with a_p .

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- ¹ Jacchia, L. G., *Nature*, **183**, 1662 (1959).
- ² Jacchia, L. G., and Slowey, J., *J. geophys. Res.*, **69**, 905 (1964).
- ³ Jacchia, L. G., Slowey, J., and Verniani, F., *J. geophys. Res.*, **72**, 1423 (1967).
- ⁴ Roemer, M., *Space Res. U.K.*, **11**, 965 (1971).
- ⁵ DeVries, L. L., *Space Res. U.K.*, **12**, 777 (1972).
- ⁶ DeVries, L. L., *Space Res. U.K.*, **12**, 867 (1972).
- ⁷ Sugiura, M., and Cain, S., *NASA Technical Note D5748* (1970).
- ⁸ Cole, K. D., *Aust. J. Phys.*, **15**, 223 (1962).
- ⁹ Wentworth, R. C., McDonald, W. M., and Singer, S. F., *Bull. Am. phys. Soc., Ser. II*, **4**, 7 (1959).
- ¹⁰ Stuart, G. W., *Phys. Rev. Lett.*, **2**, 417 (1959).
- ¹¹ Dessler, A. J., and Parker, E. N., *J. geophys. Res.*, **64**, 2239 (1959).
- ¹² Pröls, G. W., *Planet. Space Sci.*, **21**, 983 (1973).
- ¹³ Cahill, L. J., *J. geophys. Res.*, **71**, 4505 (1966).
- ¹⁴ Frank, L. A., *J. geophys. Res.*, **72**, 3753 (1967).
- ¹⁵ Cahill, L. J., *J. geophys. Res.*, **75**, 3778 (1970).
- ¹⁶ Moritz, J., *Z. Geophys.*, **38**, 701 (1972).
- ¹⁷ Mizera, P. F., and Blake, J. B., *J. geophys. Res.*, **78**, 1058 (1973).
- ¹⁸ Sugiura, M., *J. geophys. Res.*, **77**, 6093 (1972).
- ¹⁹ May, B. R., and Miller, D. E., *Planet. Space Sci.*, **19**, 39 (1970).

African Swells, Magmatism and Plate Tectonics

WRIGHT^{1,2} has revived Bailey's lateral compression model³ for the generation of Africa's topographic swells⁴⁻⁶ and the related igneous activity. Here I discuss some flaws in this approach, many of which persist in spite of its translation into plate-tectonic language.

Bailey regarded the gross "basin and swell" topography of Africa as the result of lateral compression of the continent, and he suggested that such regional updoming of the crust can bring about sufficient relief of lithostatic loading to cause melting in the deep crust (or below), to produce the characteristic East African igneous activity. Wright's variant¹ attempts a marriage between this scheme and modern global tectonics: here the entire lithosphere plate undergoes buckling, which is attributed to lateral stress attending opposed spreading at the mid-Atlantic and mid-Indian Ocean ridges².

To propose that pressure relief in the deepest parts of the crust is responsible for melting³ clearly requires that the crust alone should suffer warping, the layer beneath being relatively free of lateral stress and deformation: pressure relief can only occur at the junction between deformed and undeformed layers. Such a suggestion implies a degree of decoupling between the crust and underlying lithosphere at odds with modern global tectonics, which emphasises the integrity of the entire lithosphere layer, and it is hard to think of a mechanism by which the crust alone could be subject to compression.

If, on the other hand, one considers that the lithosphere layer as a whole undergoes buckling¹, similar arguments restrict the possibility of load relief and melting to the base of the plate, at the lithosphere-asthenosphere interface. To suggest that significant pressure relief can be induced here by plate warping assumes an order of rigidity in the asthenosphere which is questionable. Fennoscandian and Hudsonian postglacial uplift rates are known to be of the order of 1 cm yr^{-1} (ref. 8). It seems, therefore, that asthenosphere flow can be rapid enough to compensate a lithosphere uplift taking place at this rate, which is nearly two orders of magnitude greater than the mean rate of doming uplift in East Africa (5,000 foot in $\sim 1.5 \times 10^7$ yr according to ref. 4, page 1056). Since there is no evidence of deep partial melting associated with the postglacial relief of shield areas, one may argue that the probability of its occurring through compressional warping of similar terrains in Africa is insignificant. There is evidence that uplift in East Africa has been a discontinuous process, and it is arguable that intermittent spells of rapid uplift may have sustained melting, but the data above suggest that uplift rates 100 times greater than

the observed average are required before melting is remotely possible. Such rates seem unlikely.

There is evidence too that lateral compression is not capable of producing Africa's swells. Ramberg and Stephansson⁹ have analysed the effect of gravity on the maximum width of compressional warp structures, and have calculated an upper 'wavelength' limit of the order of 10 km. Comparison with the scale of the African features (200 to 500 km or more) points to the operation of some other mechanism. Moreover, the presence of tensional rift structures astride the swells belies the involvement of compression¹⁰.

There are several petrological observations bearing on partial melting models. Bailey proposed that East Africa's abundant salic lavas derive from the partial fusion of deep-crustal rocks of alkali basalt affinity. Leaving aside the efficiency of pressure relief in producing melting⁷, this idea fails to explain the coexistence at the surface of salic and basaltic volcanism, unless locally complete fusion is advocated. The origin of the basaltic reservoir and the reasons for its confinement to deep levels are unexplained.

Wright emphasises the mantle as the source of volcanism. There is, however, little petrological evidence that liquids as fractionated as trachyte or phonolite can form by fusion of ultramafic compositions. (If that were possible, why should the proportion of salic lavas be so much lower in oceanic islands than in the continents?) Rare high pressure xenoliths in trachytic rocks indicate high pressure fractionation² but not necessarily an origin by direct partial fusion of mantle rocks¹¹. They are equally consistent with fractional crystallisation of bodies of mafic liquid confined near the base of the crust, a model which explains a variety of phenomena¹². Moreover, any partial fusion scheme predicts that low-temperature melts such as trachyte should be sweated off from the mantle substantially earlier than basaltic liquids, a generalisation which is hard to reconcile with the simultaneous or alternating availability of both end members in many alkaline provinces.

I have assembled arguments showing that warping by lateral compression is at best an improbable cause of continental alkaline magmatism. The topography of Africa is now widely regarded as the product of heat flow anomalies in the upper mantle^{6,7}, from which the igneous activity where present may also be derived. I have argued recently¹² that the overabundance of salic magmas in the alkaline provinces of Africa and elsewhere, far from indicating highly specialised mechanisms for magma generation beneath the continents, merely reflects the influence of low-density continental crust on the transport of magmas of different types to the surface or present erosional levels. Thus the abundance anomaly which Bailey³ and Wright¹ seek to explain may only be superficial, and there is little reason to adhere to a model of magma genesis which fits so few of the data now available.

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¹ Wright, J. B., *Nature*, **244**, 565 (1973).

² Wright, J. B., *Geol. Mag.*, **107**, 125 (1970).

³ Bailey, D. K., *J. geophys. Res.*, **69**, 1103 (1964).

⁴ Holmes, A., *Principles of Physical Geology* (Nelson, 1965).

⁵ LeBas, M. J., *Nature*, **230**, 85 (1971).

⁶ Burke, K., and Wilson, J. T., *Nature*, **239**, 387 (1972).

⁷ Harris, P. G., *Tectonophysics*, **8**, 427 (1969).

⁸ Bott, M. H. P., *The Interior of the Earth* (Arnold, 1971).

⁹ Ramberg, H., and Stephansson, O., *Tectonophysics*, **1**, 101 (1964).

¹⁰ King, B. C., in *African Magmatism and Tectonics* (edit. by Clifford, T. N., and Gass, I. G.), 263 (Oliver and Boyd, 1970).

¹¹ Price, R. C., and Green, D. H., *Nature phys. Sci.*, **235**, 133 (1972).

¹² Gill, R. C. O., *Nature phys. Sci.*, **242**, 41 (1973).

Behaviour of the Earth's Palaeomagnetic Field from Small Scale Marine Magnetic Anomalies

CERTAIN areas of the ocean crust exhibit a high resolution recording of the magnetic field history. A recent survey¹ of the Gorda-Juan de Fuca Rise area in the North Pacific displays short wavelength (10 to 20 km), low amplitude (40 to 80 gamma) features superimposed upon the larger scale (30 to 200 km, 200 to 800 gamma) magnetic anomaly pattern of Heirtzler *et al.*². Many of these small scale anomalies form lineations which are parallel to the major magnetic lineations of Heirtzler *et al.* Sequences of small scale anomalies form characteristic patterns within intervals previously thought to be of constant polarity. We have identified two of these patterns that we observed in the North Pacific on profiles from the South Pacific and South-east Indian Oceans. Because of their global distribution, we conclude that the small scale anomalies are due to time variations of the Earth's magnetic field. That is, these features record either short (less than 3×10^4 yr) polarity reversals or fluctuations in the intensity of the dipole moment, perhaps with periods greater than 3×10^4 yr.

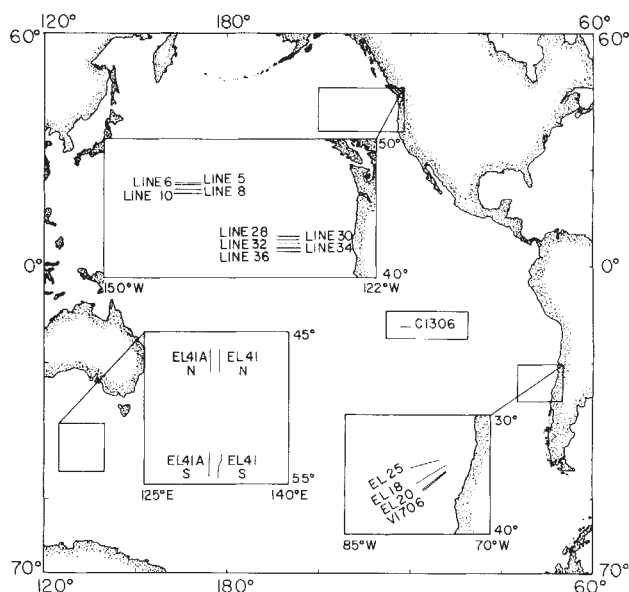


Fig. 1 Locations of profiles in Figs 2 and 3. Track prefixes: C, RV Conrad; V, RV Vema; EL, USNS Eltanin; LINE refers to Surveyor Seamap profiles.

The two sequences of small scale anomalies we studied in the North Pacific survey were between anomalies 12 and 13 (half-spreading rate 4.5 cm yr^{-1}) and between anomalies 5 and 5A (half-spreading rate 3.0 cm yr^{-1}): see Figs 2 and 3. On the east flank of the Chile Rise (half-spreading rate 4.5 cm yr^{-1}) the lineated small scale features between anomalies 12 and 13 show the same character as in the North Pacific survey area (Fig. 2). Similarly, the small scale features between anomalies 5 and 5A in the North Pacific can be recognised on magnetic profiles of both flanks of the South-east Indian Ridge (half-spreading rate 2.8 cm yr^{-1} on north flank; 2.3 cm yr^{-1} on south flank). These features can also be identified on the Conrad 1306 track (Fig. 3) which crosses anomaly 5 and 5A in the Central Pacific in an area of relatively fast spreading (half-spreading rate 6.0 cm yr^{-1}). This profile contains additional detail which may represent actual field behaviour recorded with higher resolution because of the fast spreading rate. It would be beneficial to conduct a survey of this area to determine whether this detail is repeated in other profiles.

To facilitate the correlations, we have phase shifted the various profiles according to the method of Schouten and McCamy³. The amount of phase shift is noted by the parameter $\Delta\theta$ in Figs 2 and 3.

The detailed behaviour of the Earth's magnetic field is not well known. The original reversal time scale of Heirtzler *et al.* identified no polarity events of duration less than 3×10^4 yr. Research on the magnetisation of rocks^{4,5} and high sedimentation rate cores^{6,7} however, suggests the existence of at least six short (less than 3×10^4 yr) polarity events during the past 2.4 m.y. Others^{2,8,9} have correlated some of these events with small scale anomalies on marine magnetic profiles. Blakely and Cox¹⁰ identified six additional short (less than 6×10^4 yr) polarity events in the early Cretaceous from magnetic profiles. Blakely¹¹ is revising the Heirtzler time scale between anomalies 5 and 6. He has identified many of the same small scale features that we have between anomalies 5 and 5A.

McElhinny¹² points out the possibility that some of the events identified from the magnetisation of rocks and high sedimentation rate cores may be due to polar excursions of the dipole field rather than complete polarity reversals. In addition, near-bottom magnetic surveys suggest that there are variations in the magnetisation intensity of the ocean crust due either to fluctuations in palaeomagnetic field intensity or

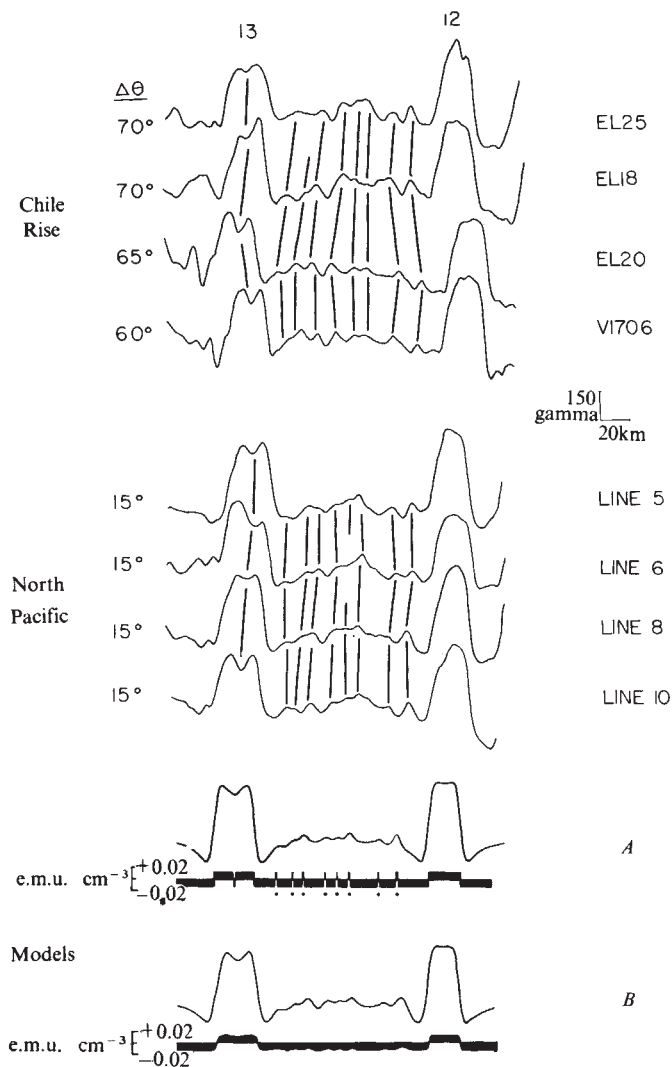


Fig. 2 Correlations of small scale features between anomalies 12 and 13. Models explained in text. Darkened figures represent magnetisation distributions. Dots in model A indicate locations of possible new events. Model A parameters: depth, 4.5 km; layer thickness, 0.5 km; smoothing (σ), 0.5 km; $\Delta\theta$, phase shift parameter³.

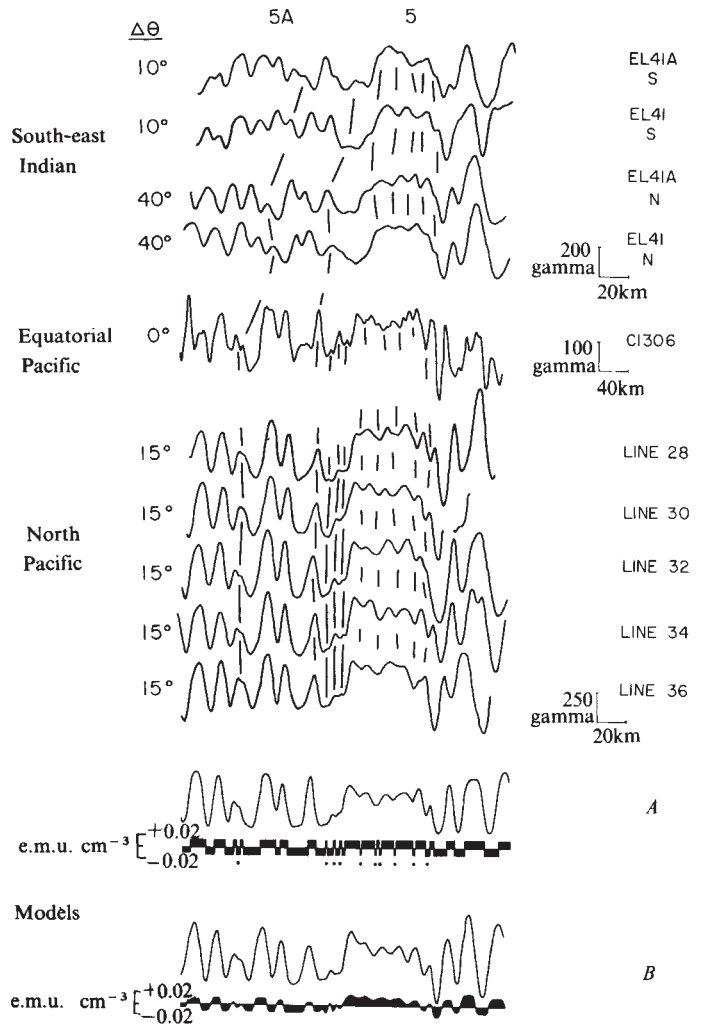


Fig. 3 Correlations of small scale features between anomalies 5 and 5A. Model parameters: depth, 3.5 km; layer thickness, 0.5 km; smoothing (σ), 0.5 km; $\Delta\theta$, phase shift parameter³.

variations in petrology^{13,14}. Others^{15,16} have noted the existence of dipole field intensity fluctuations with a periodicity of 10^4 yr.

Surface marine magnetic profiles lack sufficient detail to discriminate between a crustal magnetisation distribution due to short periods of reversed polarity and one due to intensity fluctuations in the palaeomagnetic field. Models A and B (Figs 2 and 3) demonstrate that both hypotheses can account for the observed small scale anomalies. Model A represents the 'reversal model' in which the field maintains constant intensity during periods of constant polarity. The duration of the polarity transition is assumed to be 5×10^3 yr (ref. 17). Model B represents the 'intensity fluctuation model' in which the intensity varies irregularly during periods of constant polarity. The models do not duplicate any one profile exactly. Instead they approximate the general character of all the profiles. The model magnetisation distributions have been smoothed with a Gaussian function to simulate the effect of such processes as dike injection and extrusion of lavas. The amount of smoothing is measured by the standard deviation, σ , of the Gaussian function^{3,10}. Based on the results of near-bottom surveys^{18,19} we assumed a standard deviation, σ , of 0.5 km.

Model A shows the possible location of short polarity reversals required to duplicate the observed anomaly pattern. These new events are almost all less than 3×10^4 yr in duration. We have inserted eight new events between anomalies 12 and 13 and 10 new events between anomalies 5 and 5A.

The existence of additional short period events in the time scale has been predicted by Cox¹⁵ on the basis of a stochastic model. Assuming these features are due only to polarity reversals, the insertion of these new events changes the observed distribution of polarity interval lengths. Since these small scale features are seen throughout the time covered by the North Pacific survey area (40 m.y. BP to present), this suggests that the mean rate of polarity reversals may be considerably greater than previously estimated^{2,15}, perhaps by a factor of two. During intervals previously thought to be of constant polarity, the field may have behaved like a monostable oscillator, favouring one polarity more than the other. In particular, this behaviour may characterise the early Cretaceous where the Heirtzler *et al.* time scale identifies many long periods of constant polarity. Such monostable behaviour may be incompatible with a simple stochastic model like the Poisson process.

Model *B* demonstrates an alternative hypothesis that these small scale features may be due to intensity fluctuations within periods of constant polarity. The features were modelled by fluctuation with periods of 3×10^4 yr to 2×10^5 yr and amplitudes of 15% or more of the mean field intensity. The magnetisation shown in model *B* is not a unique representation of the actual field behaviour. The small scale features could be modelled by shorter period fluctuations of greater amplitude or the observed features may be artefacts of an even more complicated behaviour.

We considered the possibility that the intensity of fluctuations in model *B* might be explained by variations in the dipole moment of the field, variations in the intensity of the non-dipole field or variations in the pole position of the dipole field. The high coherence of the small scale anomalies recorded in different hemispheres over millions of years eliminates the possibility that they are due to variations in the non-dipole field. Variations in the pole position of the dipole field would have produced a phase difference of 180° between the variations observed in the Northern Hemisphere and Southern Hemisphere. This relative phase difference is not apparent in our profiles. Therefore, we conclude that the intensity fluctuations of model *B* would be due to variations in the dipole moment.

Near-bottom magnetometer profiles in areas where small scale lineated anomalies are observed at the surface may help to resolve the nature of the field behaviour responsible for the anomalies.

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- ¹ IDOE 1971 Surveyor Seemap (NOAA Environmental Data Service, Washington, DC, 1971).
- ² Heirtzler, J. R., Dickson, G. O., Herron, E. M., Pitman, III, W. C., and Le Pichon, X., *J. geophys. Res.*, **73**, 2219 (1968).
- ³ Schouten, H., and McCamy, K., *J. geophys. Res.*, **77**, 7089 (1972).
- ⁴ Bonhommet, N., and Babkine, J., *C.r. hebdo. Séanc. Acad. Sci. Paris*, **264**, 92 (1967).
- ⁵ McDougall, I., and Chamalaun, F. H., *Nature*, **212**, 1415 (1966).
- ⁶ Smith, J., and Foster, J. H., *Science*, **163**, 565 (1969).
- ⁷ Nakajima, T., Yaskawa, K., Natsuhara, N., Kawai, N., and Horie, S., *Nature phys. Sci.*, **224**, 8 (1973).
- ⁸ Emilia, D. A., and Heinrichs, D. F., *Marine geophys. Res.*, **1**, 436 (1972).
- ⁹ Blakely, R. J., and Cox, A., *J. geophys. Res.*, **77**, 4339 (1972).
- ¹⁰ Blakely, R. J., and Cox, A., *J. geophys. Res.*, **77**, 7065 (1972).
- ¹¹ Blakely, R. J., *J. geophys. Res.* (in the press).
- ¹² McElhinny, M., *Paleomagnetism and Plate Tectonics* (Cambridge University Press, 1973).
- ¹³ Luyendyk, B. P., *J. geophys. Res.*, **74**, 4869 (1969).

- ¹⁴ Larson, R. L., Larson, P. A., Mudie, J. D., and Spiess, F. N., *J. geophys. Res.* (in the press).
- ¹⁵ Cox, A., *Science*, **N.Y.**, **163**, 237 (1969).
- ¹⁶ Smith, P. J., *Geophys. J.*, **12**, 321 (1967).
- ¹⁷ Opdyke, N. D., Kent, D. V., and Lowrie, W., *Earth planet. Sci. Lett.* (in the press).
- ¹⁸ Atwater, T., thesis (Univ. California, San Diego, 1972).
- ¹⁹ Klitgord, K. D., Mudie, J. D., and Normak, W. R., *Geophys. J. R. astr. Soc.*, **28**, 35 (1972).

North Sea Geothermal Gradients

REGIONAL studies of geothermal gradient patterns obtained from bottom hole temperature measurements in deep oil exploration boreholes have been the subject of several recent papers¹⁻³. Harper¹ has produced approximate gradients in a study of the North Sea by the direct use of bottom hole temperatures routinely taken during the logging of exploration wells. These temperatures, which are taken with a maximum mercury-in-glass thermometer at a point typically 30 foot from the bottom of the hole, are, in general, less than the true formation temperature as a result of the cooling effect of the circulating drilling fluid.

The effect of such a thermal disturbance in a borehole may be approximated by a constant line heat source in a uniform infinite medium with a solution as a function of time, as derived by Lachenbruch and Brewer⁴. That solution, for a fixed depth in a borehole, is

$$T(t_2) = K \log(1 + t_1/t_2) + T(\infty) \quad (1)$$

where t_1 is the time of cooling the formation by the circulating fluid, t_2 is the interval from cessation of cooling to temperature measurement, $T(t_2)$ is the observed temperature at time t_2 , $T(\infty)$ is the true formation temperature and K is a constant. Thus where multiple values of $T(t_2)$, t_1 , and t_2 are known, equation (1) may be applied as a semi-log plot of $T(t_2)$ against $(1 + t_1/t_2)$ to produce accurate formation temperatures at the

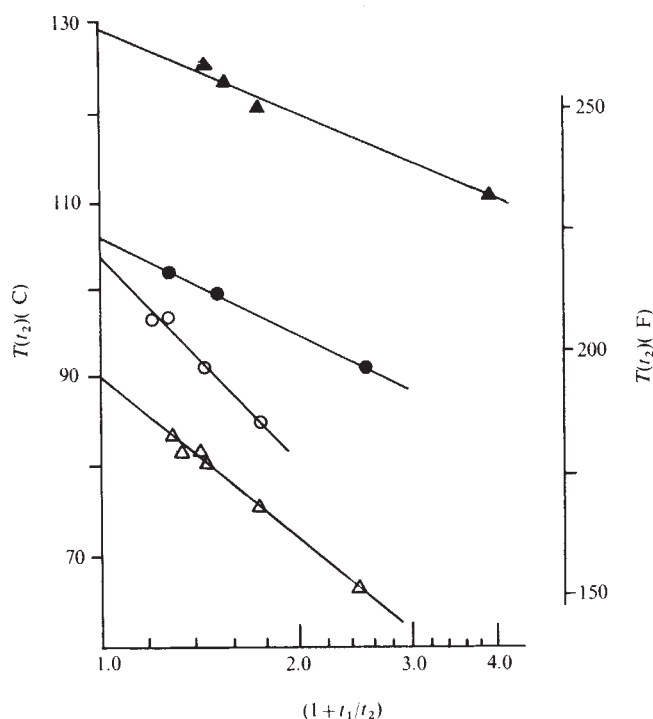


Fig. 1 Thermal recovery of four North Sea exploration boreholes. The temperature intercept is the true formation temperature. $\blacktriangle$, BP, UK; $\bullet$, Conoco, UK; $\circ$, Amoco, UK; $\triangle$, BP, UK.

linear axis intercept as was recently shown by Timko and Fertl⁵.

Bottom hole temperatures from fifty-nine North Sea oil exploration wells, forty in the UK sector, fifteen in the Norwegian, and four in the Dutch, were studied and true formation temperatures calculated by the above method. Figure 1 is a semi-log plot for four typical wells. This method was also applied to a further forty-four results from the previous study by Harper¹. These were additional data without t_1 values and it was, therefore, necessary to estimate this parameter statistically. The interval t_1 , the total time of cooling, is the sum of the time taken to drill the final 30 foot of hole plus any additional time during which fluid circulates after drilling ceases. A log linear relation for drilling rate against depth was observed, while circulation time approximated a normal distribution. These results, based on data from forty-six North Sea wells, allow a satisfactory estimation of t_1 and provide a basis for the application of equation (1).

The determination of all geothermal gradients incorporated the subtraction of sea bottom temperatures from the true formation temperatures with all depths being calculated from sea bottom. Defant⁶ showed that the bottom temperature of the central North Sea varies both with water depth and season of the year. Figure 2 shows the transformation of Defant's results to a mean annual temperature at sea bottom as a function of water depth. This result was combined with the true formation temperatures for the calculation of gradients. Geothermal gradients calculated from bottom hole temperatures uncorrected for the thermal disturbance of the drilling fluid would typically be lower than true gradients by 10–15%.

A geothermal gradient contour map, Fig. 3, is based on the total one hundred and three determinations with individual results being averaged over petroleum lease blocks which are 8 by 12 miles in the UK sector and somewhat larger in the others. As stated by Harper¹ and confirmed here, the regional trends seem to be governed by the major structural elements. The major sedimentary basins, English, SW Netherlands, German, Norwegian, East Shetlands, Moray Firth, and Midland Valley are all areas of high geothermal gradients with values locally reaching $44^\circ \text{C km}^{-1}$. Structural highs including Ringkobing-Fyn, Norwegian Platform, Mid North Sea, Mid Netherlands, and Grampian are characterised by low gradients which, south of Norway, approach $18^\circ \text{C km}^{-1}$. A geothermal high confirmed by four wells, located at approximately 55°N , 00°E , seems to confirm Armstrong's⁷ opinion of the probable presence of Carboniferous coal-bearing rocks at a distance of one hundred miles from the Yorkshire coast trending ESE

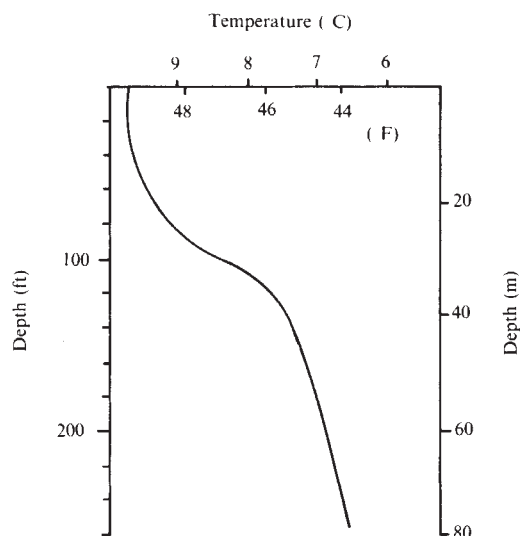


Fig. 2 Mean annual North Sea bottom temperature against water depth.

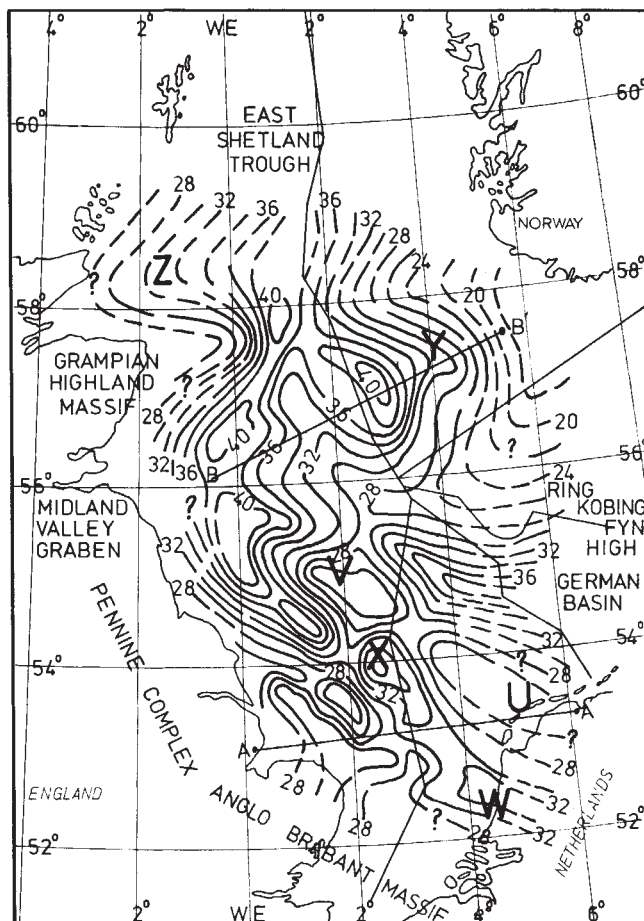


Fig. 3 Geothermal gradients, North Sea. Contour interval 2°C km^{-1} . U, Mid Netherlands Ridge (3%); V, Mid North Sea High (11%); W, S.W. Netherlands Basin (8%); X, English Basin (46%); Y, Norwegian Basin (18%); Z, Moray Firth Basin (2%). Numbers in parentheses refer to percentages of total data in each area. The remaining 12% of data is in the Western (British) extension of the Norwegian Basin.

to fifty miles from the Norfolk coast. A thin stratum of coal above the bottom hole temperature measurement point with thickness 2–3% of that of the total formation would lower the overall thermal conductivity by approximately 25% due to its very low thermal conductivity⁸, and could thus be a viable explanation of this anomaly. In addition there is a strong correlation, north of 56° , with the recently published Palaeocene isopach and base of Tertiary structure maps⁹. The coincidence of high geothermal gradients with the Tertiary depocentres reflects the lower thermal conductivity of this predominantly marine shale sequence.

Figure 4 incorporates diagrammatic cross sections of the

Table 1 Estimates of Thermal Conductivity

Section	Dominant lithology	Thermal conductivity [$\text{W m}^{-1} \text{K}^{-1}$]
Quaternary/Tertiary	Shale ¹¹	1.76 (ref. 3)
Cretaceous (including Danian)	Chalk ^{7,11}	1.84 (unpublished observation)
Jurassic	Sand/shale ^{10,11}	2.64 (ref. 3)
Triassic	Sand ^{7,10,11}	3.10 (ref. 3)
Upper Permian (Zechstein)	Evaporite/carbonate ^{7,10,11}	3.85 (ref. 12)
Lower Permian (Rotliegendes)	Sand ^{10,11}	3.10 (ref. 3)
Carboniferous	Shale/limestone ^{10,11}	1.94 (ref. 12)

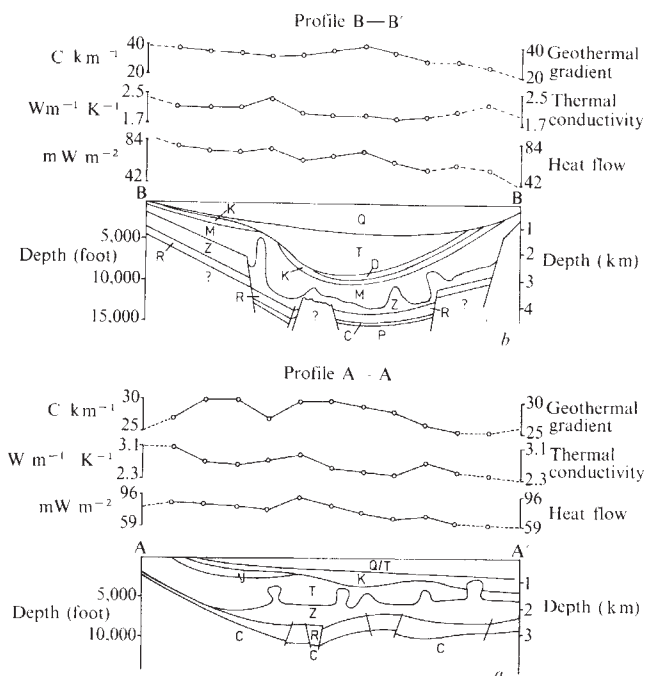


Fig. 4 *a*, Thermal and geological profiles of the southern North Sea. Geology after Armstrong⁷. Data points are samples from the contour map, Fig. 3, taken at equispaced points on profile A-A'. Q, Quaternary; T, Tertiary; D, Basal Tertiary/Upper Cretaceous (Danian); K, Cretaceous; J, Jurassic; T, Triassic; M, undifferentiated Jurassic/Triassic; Z, Upper Permian (Zechstein); R, Lower Permian (Rotliegendes); C, Carboniferous. *b*, As *a* but for central North Sea, profile B-B'.

North Sea in the positions indicated on Fig. 3 as well as the geothermal gradient, conductivity, and heat flow profiles. Estimates of thermal conductivity (Table 1) were made from published descriptions of the major lithological units of the North Sea together with the measured thermal conductivities of similar materials. Mean estimated heat flows of 75 mW m⁻² from the southern profile (A-A') and 71 mW m⁻² from the northern profile (B-B') were obtained, thus indicating no significant difference despite the higher gradients observed to the north. The values of heat flow, while slightly higher than the world mean¹³, are intermediate in terms of other European basin values¹ and in good agreement with the world-wide mean for marginal seas¹⁴. A possibly significant aspect of Fig. 4 is that both heat flow profiles show an apparent decrease towards the European continent. This is consistent with world-wide heat flow means of 61.9 mW m⁻² for post-Precambrian orogenic areas such as Great Britain and 38.5 mW m⁻² for Precambrian shields such as Norway¹⁴. Seven recent combined heat-flow production measurements in Precambrian and Permian igneous rocks in southern Norway support such a value¹⁵. But, in general, the paucity of heat flow data in this area limits any attempt to confirm this trend.

Recent oil discoveries in the North Sea such as the Forties (57° 40' N, 1° 0' E) and Ekofisk (56° 30' N, 3° 10' E) fields seem to support the theories of Klemme¹⁶ that high geothermal gradients enhance petroleum mobility, that is, migration to structural traps, to a greater degree than they adversely affect reservoir quality.

We thank the following oil companies for permission to use temperature data from their North Sea wells: Amoco, BP, Conoco and Phillips. We also thank Drs M. L. Harper and H. Y. Tammemagi for advice.

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- ¹ Harper, M. L., *Nature*, **230**, 235 (1971).
- ² Summers, W. K., *Am. Assoc. Petrol. Geol. Bull.*, **56**, 2072 (1972).
- ³ Girdler, R. W., *Phil. Trans. R. Soc.*, **A267**, 191 (1970).
- ⁴ Lachenbruch, A. H., and Brewer, M. C., *US Geol. Surv. Bull.*, **1083C**, 78 (1959).
- ⁵ Timko, J. T., and Fertl, W. M., *Wld. Oil*, **175**, 73 (1972).
- ⁶ Defant, A., *Phys. Oceanogr.*, **1**, 116 (1961).
- ⁷ Armstrong, G., *Min. Eng.*, **131**, 474 (1972).
- ⁸ Bullard, E. C., and Niblett, E. R., *Mon. Not. R. astr. Soc. geophys. Supp.*, **6**, 222 (1951).
- ⁹ Dunn, W. W., Eha, S., and Heikkila, H. H., *Oil Gas J.*, **71**, 90 (1973).
- ¹⁰ Gill, W. D., *Proc. Seventh W. Petrol. Congr., Mexico*, **2**, 211 (1967).
- ¹¹ King, R. E., *Wld. Oil*, **175**, 35 (1972).
- ¹² Clark, S. P., *Handbook of Physical Constants*, Mem. 97, *Geol. Soc. Am.*, 459 (1966).
- ¹³ Lee, W. H. K., and Uyeda, S., *Am. geophys. Monog. Ser.*, **8**, 87 (1965).
- ¹⁴ Lee, W. H. K., and Uyeda, S., *Am. geophys. Monog. Ser.*, **8**, 147 (1965).
- ¹⁵ Swanberg, C. A., Simmons, G., Chessman, M. D., Gronlie, G., and Heier, K. S., *Trans. Am. geophys. Union*, **54**, 464 (1973).
- ¹⁶ Klemme, H. D., *Oil Gas J.*, **70**, 76 (1972).

Quantitative Tar and Plastic Waste Distributions in the Pacific Ocean

WE report in this letter the first quantitative data on tar and plastic waste distributions in the surface waters of the Pacific Ocean. During the Canadian Transpac-72 cruise, thirty-seven surface tows were made with a neuston net to collect particulate pollutants quantitatively. (The net, a Kahl Scientific model with a mouth 80 cm by 30.5 cm and a 150 µm mesh net, was towed at 4 to 5 knots for 10 to 15 min at a time, covering an average area of 1,800 m². It was rigged so that it would tow off to the side of the ship through water undisturbed by the ship's wake.) The research ship, CSS Parizeau (track shown in Fig. 1), left Tokyo on October 10, 1972, proceeded east along latitude 35° N to near the California coast, then turned north along 125° W to reach Victoria, British Columbia, on November 2, 1972.

The tar distribution is shown in Fig. 2. Tar was present in thirty of thirty-three tows along 35° N as black or brownish-black lumps up to 3 cm in diameter. Its concentration was consistently higher in the Western Pacific from Tokyo to waters north of the Hawaiian Islands with a maximum of 14 mg m⁻² wet weight and an average of 3.8 mg m⁻²—the same order of magnitude as the weight of the organisms caught in the neuston net. The concentration of tar in the Eastern Pacific was an order of magnitude lower on average. No tar was found along 125° W. These tar aggregates were inhabited by diverse biological communities: diatoms and bryozoans, usually on the smaller pieces, and blue-green algae and goose barnacles on some of the larger ones. Crabs, mostly still in the megalops stage, and, as in the Atlantic¹⁻³, the isopod *Idotea metallica* seemed to be living on the lumps. Thus, the tar is apparently not directly toxic to many species, although incorporation of small lumps into the food chain by way of surface-feeding fish and copepods has been suggested as a hazard^{1,4}.

As shown in Fig. 2, plastic is widespread, although in minute quantities. It was present in twenty-one of thirty-three tows along 35° N as small (0.1 to 0.5 cm), round, colourless pellets weighing 20 to 50 mg each. In contrast to tar, the maximum concentration of plastic was found in the Eastern Pacific (3.5 mg m⁻²). The average concentration was 0.3 mg m⁻². The maximum number of pieces observed was sixty-two, corresponding to 34,000 pieces km⁻². None was found along 125° W. Smaller plastic pieces did not appear to support

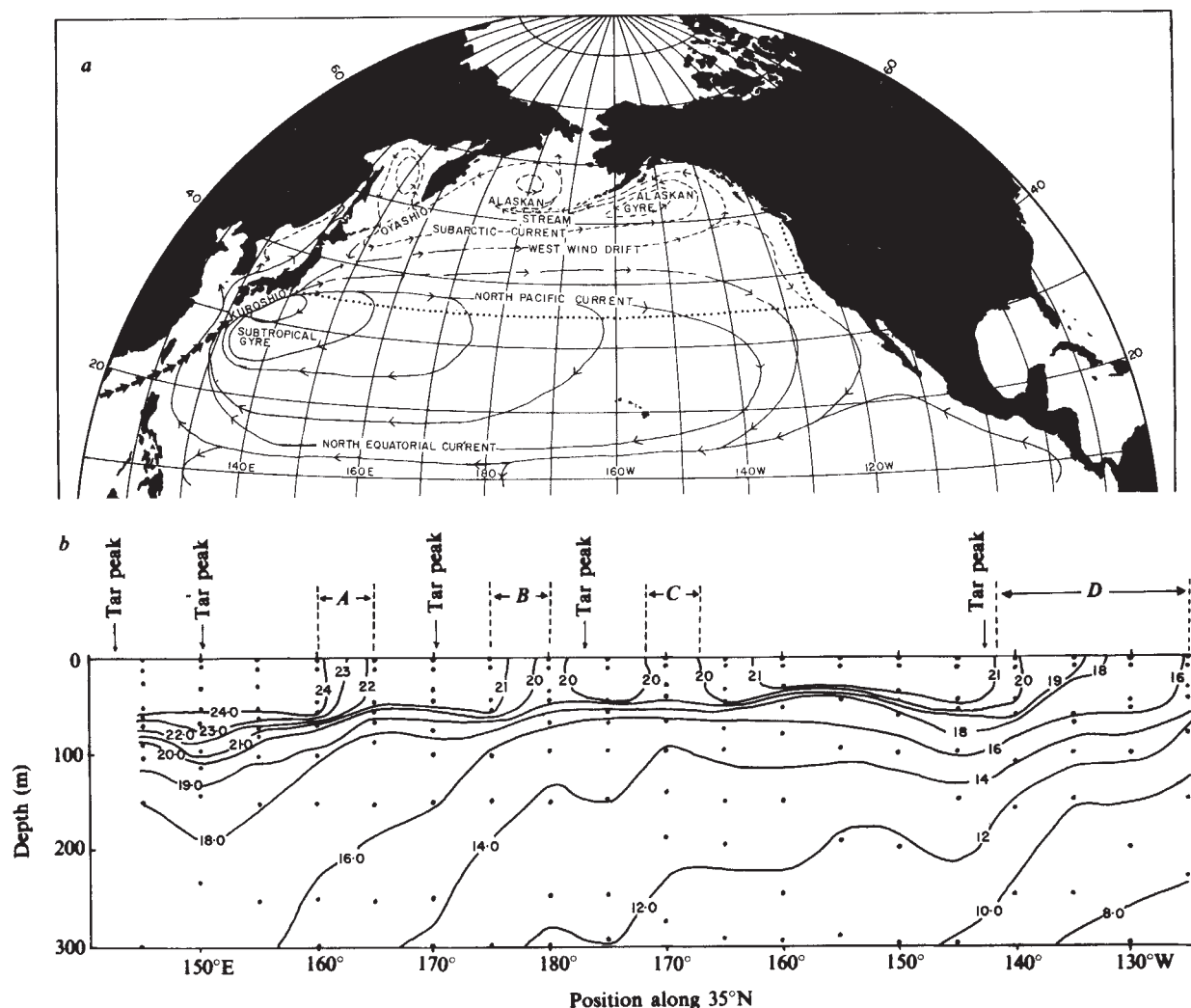


Fig. 1 *a*, Track of the CSS Parizeau on Transpac-72 cruise (·····), the surface transport in the North Pacific Ocean⁹ (cold currents, ---; warm currents, —), and the Persian Gulf-Japan tanker route (heavy arrows). *b*, Temperature distribution (°C) in the surface waters along 35° N in the Pacific Ocean. The zones of influx of cold subarctic water (A, B, C, D) and the locations of the peak concentrations of tar are indicated.

a fouling community, although larger ones had growths similar to that on tar lumps of the same size.

Some paper, elastic bands and wood were present in the first few tows off Japan (up to 500 miles from Tokyo). None was found, however, in the remaining 6,000 mile journey.

The persistent tar distribution may result from several factors: likely sources from marine oil transport, augmented by accidental spills, prevailing wind transport and surface circulation.

First, an order of magnitude difference in 'possible-source' volumes of oil shipments to Japan and the west coast of the United States correlates well with a similar order of magnitude difference in tar for Western and Eastern Pacific waters, assuming that persistent tar originates mainly from tanker washings. In the North-western Pacific, 6,000 t.b.d. (thousand barrels per day) of crude oil were transported by tankers in 1971, 90% going to Japan along the Persian Gulf-Japan tanker route (Fig. 1). In the North-eastern Pacific, only 600 t.b.d. of seaborne crude oil were handled. The locations of the tanker routes do not, however, agree with the detailed distribution pattern of pelagic tar. (Information on oil tanker routes is from ref. 5.)

Second, prevailing winds are most effective in transporting surface pollutants. In general, a high concentration of tar in the Western Pacific between 150° E and 175° W and in the Eastern Pacific at 143° W is consistent with the mean annual wind stress over the North Pacific⁶. The wind stress tends to

move surface pollutants eastwards in the Western Pacific to about 170° W where both a change to a southward direction and a weakening in wind stress takes place. The 143° W peaks of tar and plastic are in a region of zero annual wind stress, where accumulation of both tar and plastic is expected. This does not explain the absence of plastic in the Western Pacific, which possibly reflects the liberal input from the Hawaiian Islands relative to Japan.

Third, surface circulation also affects the long term distribution of surface pollutants. The cruise track of Transpac-72 at 35° N crosses an area influenced by both warm subtropical water and colder subarctic water. The fast moving warm Kuroshio Extension current flows east from 36° N, 140° E, in a remarkable meandering path up to about 160° E, where it transforms into the weak, broad North Pacific Current⁷. The waters further east undergo many seasonal and spatial variations due to mixing and incursion of cold subarctic water across the normal boundaries of the warm subtropical gyre water⁸. East of 140° W, the water is mainly of subarctic origin. The probable paths of intrusion of subarctic water can be inferred from a summary of North Pacific transport⁹ and from the temperature distribution, both shown in Fig. 1: (A) 160° E to 165° E at about 22° C; (B) 175° E to 180° W with temperature slightly below 20° C; (C) 173° W to 167° W at about 19.5° C; (D) east of 142° W with very rapid drop in temperature from 21° C to 16° C at 125° W. (Figure 1 was modified to include recent data on the North Pacific circulation supplied

BIOLOGICAL SCIENCES

Replication of Yeast Chromosomal DNA

THE study of replication in higher eukaryotes has been hampered by the large size of the DNA molecules in their chromosomes. The yeast *Saccharomyces cerevisiae* is a eukaryote with very little DNA per haploid nucleus (9×10^9 daltons)¹. This has facilitated measurement of intact yeast chromosomal DNA by sedimentation velocity² and electron microscopy³. The results indicate that yeast chromosomes are similar in size to those of viruses and bacteria, each chromosome containing a single DNA duplex ranging from 10^8 to 10^9 daltons. More than 95% of the chromosomal DNA molecules isolated from asynchronous cultures are simple linear structures, but the few branched structures are good candidates for replication intermediates³. We have used two experimental approaches which provide evidence that these exceptional molecules are, indeed, replication intermediates. Characterisation of these

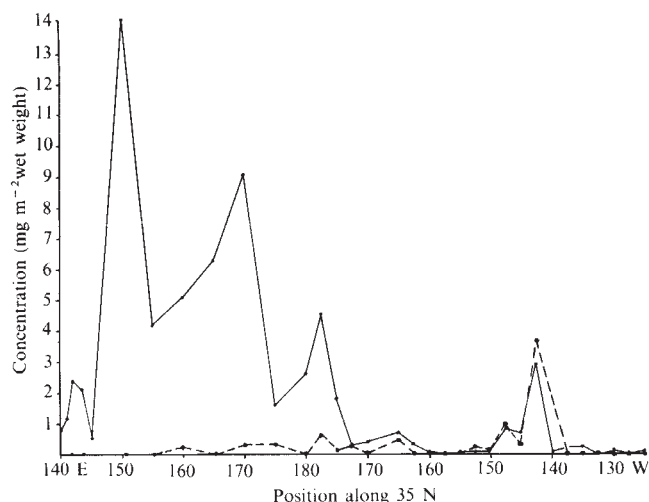


Fig. 2 Distribution of tar (—) and plastics (---) along 35° N in the Pacific Ocean. Tows covered an area of $1,800 \pm 300$ m².

by R. E. Thomson.) The tar peaks at 150° E, 170° E, 178° W and 143° W seem to be in the slack, warmer subtropical water separated by the above zones of colder subarctic water, which have a low tar content. It is worthwhile to note that the time series at ocean weather station 'Papa' (145° W, 50° N) in subarctic water shows zero tar values (unpublished data of Ocean Chemistry Division, Marine Sciences Directorate, Pacific Region, Department of the Environment, Victoria, BC, Canada). Our observations are also in agreement with known features in the Atlantic¹⁰ which show much higher concentrations of tar in the subtropical Sargasso Sea.

Due to the highly complex interaction of these three factors in the ocean, we cannot explain the waste distribution satisfactorily from one set of data. Our values in the Pacific, combined with those obtained in portions of the Atlantic and in the Mediterranean by other workers^{1,2,10,11}, do, however, indicate that the tar and plastic wastes are widespread on the surface of the oceans.

We thank William Heath for biological identifications, the captain and crew of the CSS Parizeau for help with the sampling and Drs R. W. Stewart and R. E. Thomson of Marine Sciences Directorate, Pacific Region, for criticism.

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- Horn, M. H., Teal, J. M., and Backus, R. H., *Science*, N.Y., **168**, 245 (1970).
- Polekarpov, G. G., Yegorov, V. N., Ivanov, V. N., Tokareva, A. V., and Feleppov, I. A., *Priroda*, No. 11 (1971).
- Herring, P. J., *J. mar. Biol. Assoc. U.K.*, **49**, 766 (1969).
- Conover, R. J., *J. Fish. Res. Bd. Can.*, **28**, 1327 (1971).
- International Petroleum Encyclopedia* (Petroleum Publishing Co., Tulsa, Oklahoma), 18 (1972).
- Malkus, J. S., in *The Sea*, **1**, 182 (Interscience, New York, 1963).
- Kawai, H., in *Kuroshio, Physical Aspects of the Japan Current* (edit. by Stommel, H., and Yoshida, K.), 235 (University of Washington Press, Seattle, 1972).
- Reid, J. L., *Limnol. Oceanogr.*, **7**, 287 (1962).
- Sverdrup, H., Johnson, M. W., and Fleming, R. F., *The Ocean*, 727 (Prentice-Hall, Englewood Cliffs, 1942).
- Morris, B. F., and Butler, J. N., *Proc. Conf. Prevention Control Oil Spills*, 521 (Washington, March 13–15, 1973).
- Carpenter, E. J., and Smith, jun., K. L., *Science*, N.Y., **175**, 1240 (1972).

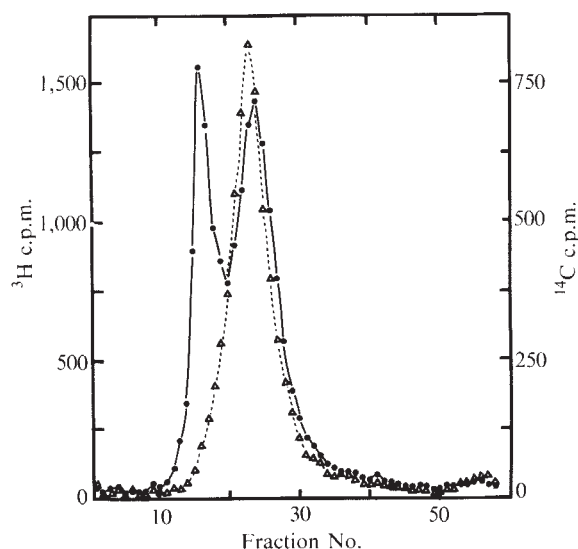


Fig. 1 Density profile of yeast DNA from a culture shifted from dense to light medium. A derivative of A364A D5 (ref. 13), A364A D5 U⁺A⁺, which does not require uracil and adenine was grown for many generations at 30° C in ¹⁵N-D₂O medium containing 0.25 μCi ml⁻¹ 2-¹⁴C-uracil (New England Nuclear Corp., 0.5 μCi μg⁻¹). The medium contains (per litre) 1.45 g Difco yeast nitrogen base (without amino acids or (NH₄)₂SO₄), 10 g succinic acid, 6 g NaOH, 50 mg tyrosine, 20 g glucose, 100 mg ¹⁵NH₄Cl (Isomet, Oakland, New Jersey) and 1 litre D₂O (Bio-Rad Laboratories, Richmond, California). The cells were filtered on Millipore HA filters and resuspended in Y-minimal medium (6.79 g Difco yeast nitrogen base (without amino acids), 10 g succinic acid, 6 g NaOH l⁻¹) supplemented with 20 g glucose and 50 mg tyrosine l⁻¹ and containing 10 μCi ml⁻¹ 6-³H-uracil (New England Nuclear Corp., 250 μCi μg⁻¹). After incubation for 100 min, cells were collected by centrifugation and spheroplasted as described previously² at a concentration of 10⁸ cells ml⁻¹. Spheroplasts were washed by centrifugation at 4° C with a solution containing 1 M sorbitol, 0.1 M sodium citrate and 0.01 M Na EDTA, pH 5.8, suspended in the same solution, and 0.3 ml (about 6 × 10⁷ spheroplasts) was layered over 0.1 ml of 10 mg ml⁻¹ pronase (previously treated at 70° C for 10 min) on top of 7 ml CsCl (Harshaw Chemical Co., Solon, Ohio) solution (η=1.4040) containing 0.01 M Tris-Cl (pH 8) and 0.01 M Na-EDTA (pH 8) in a centrifuge tube. The spheroplasts were lysed by the addition of 0.1 ml 10% sarkosyl. After 30 min at room temperature, gradients were overlaid with mineral oil and centrifuged in a Spinco 50 Ti rotor at 20° C and 33,000 r.p.m. for 60 h. Gradients were collected from the bottom of the tube through a 13 gauge needle, which had been boiled in 0.1 M EDTA, at a rate of less than 0.5 ml min⁻¹. DNA radioactivity was measured on 50 μl portions of gradient fractions as described previously². ●, ¹⁴C; △, ³H.

molecules by electron microscopy indicates that yeast DNA replication is similar to higher eukaryotic DNA replication in two important respects. First, most and possibly all initiations are internal. Second, many chromosomes contain more than one replication unit.

Material enriched in replicating structures can be obtained by transferring cells from isotopically dense to isotopically light medium and banding the DNA in a density gradient. Partially replicated molecules have buoyant densities intermediate between unreplicated and completely replicated molecules. Figure 1 shows the buoyant density profile of yeast DNA prepared from a culture grown in dense medium and transferred to isotopically light medium 100 min before collection. The peak containing only ^{14}C radioactivity corresponds to fully dense DNA and the peak containing both ^3H and ^{14}C radioactivity contains hybrid density DNA. When DNA from the region between the peaks (fractions 19,

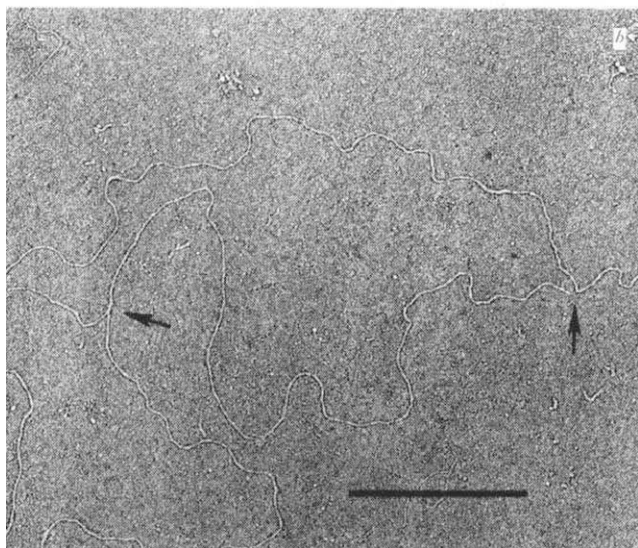
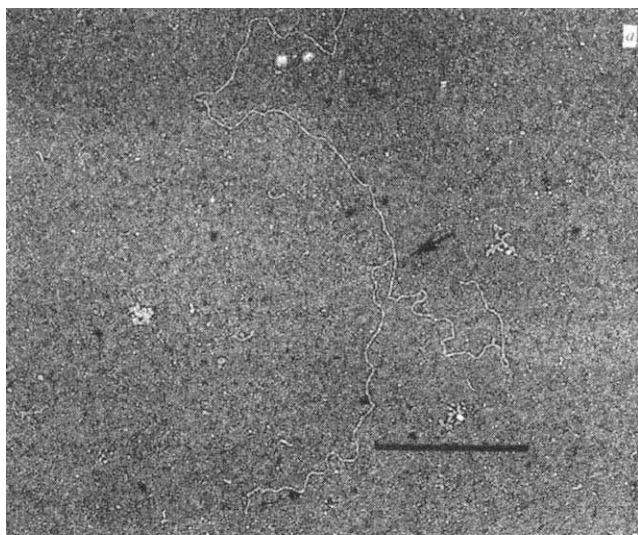


Fig. 2 Electron micrographs of portions of yeast DNA replication intermediates. DNA from CsCl gradients (Fig. 1) or sucrose gradients (Fig. 3) was spread using a modification of the aqueous procedure of Davis, Simon and Davidson⁹ which has been described in detail³. Molecules were photographed using a Philips EM 300 electron microscope. Contour lengths in cm were determined using a Keuffel and Esser map measurer and were converted to microns by comparing the contour lengths of yeast DNA to the contour length of intact bacteriophage T4 DNA³ spread under the same conditions. T4 DNA has a well characterized molecular weight (1.2×10^8) and contour length (52 μm)⁹. *a*, A Y-shaped fork with arms 2.2 μm long. *b*, A bubble with branches between the forks 5.5 μm long. The bars represent 1 μm . The arrows indicate forks.

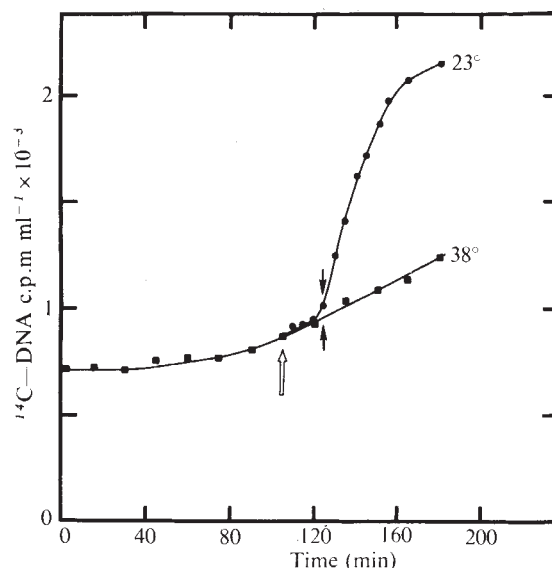


Fig. 3 Pattern of DNA synthesis in a synchronous culture. A culture of the temperature-sensitive cell division cycle mutant *cdc 7* (strain 4008, obtained from L. H. Hartwell) was grown at 23° C in Y-minimal medium supplemented with 20 g glucose, 0.5 g yeast extract, 20 mg uracil, 20 mg adenine, 50 mg tyrosine, 50 mg histidine, and 50 mg lysine per litre and 1 $\mu\text{Ci ml}^{-1}$ 2- ^{14}C -uracil (24 mCi mmol⁻¹, Schwarz/Mann) to a density of 2.5×10^6 cells ml⁻¹. To avoid holding cells at 38° C for a long time, the culture was synchronised by treatment with α -factor for one generation (3.5 h) as described previously¹². To remove α factor, cells were filtered on Millipore HA filters, washed with 200 to 400 ml medium at 38° C and resuspended in the original volume of medium at 38° C ($t=0$). After 105 min at 38° C (arrow), the culture was divided and half was shifted to 23° C. Twenty minutes later samples were taken from each culture (small arrows) and DNA was prepared for electron microscopy by sedimenting it away from smaller cellular components on sucrose gradients as described previously³. Incorporation of ^{14}C -uracil into DNA was determined as described previously¹³.

20 and 21) was examined in the electron microscope 31% (thirty out of ninety-seven) of the molecules seen contained either Y-shaped terminal forks or internal 'bubbles' (Fig. 2). These bubbles are similar to structures observed in λ (refs 4 and 5), T4 (ref. 6) or T7 (ref. 7) replicating DNA which result from pairs of diverging replication forks arising from single initiation sites. In molecules which were Y-shaped, two of the arms were equal in length. In the case of bubbles, the two branches between the forks were equal in length. These structures must be almost entirely composed of double stranded DNA because under the spreading conditions used single stranded DNA collapses on itself in 'bushes'⁸. However, small single-strand regions of the sort found in bacteriophage λ (ref. 10), T4 (ref. 6) and T7 (ref. 11) replication structures might not have been detected.

If these structures are associated with replication, samples from the fully dense and fully hybrid peaks should contain mostly simple linear molecules. In DNA from fraction 16 (Fig. 1), the peak of the fully dense region, seventy-nine out of eighty molecules examined were simple linear structures.

Replication intermediates should be found in cells in S phase, but not in cells at other stages in the cell cycle. To examine this we used a temperature-sensitive mutant defective in the initiation of DNA synthesis. In a culture of this mutant maintained at the non-permissive temperature (38° C), cells accumulate in the cell cycle just before DNA synthesis (unpublished results of L. M. H. and L. H. Hartwell). Figure 3 shows that when such a culture is shifted to the permissive temperature (23° C), it undergoes a synchronous round of DNA synthesis. The DNA synthesised in the culture maintained at 38° C is primarily mitochondrial DNA (unpublished results of C. S. N.). Twenty minutes after the temperature

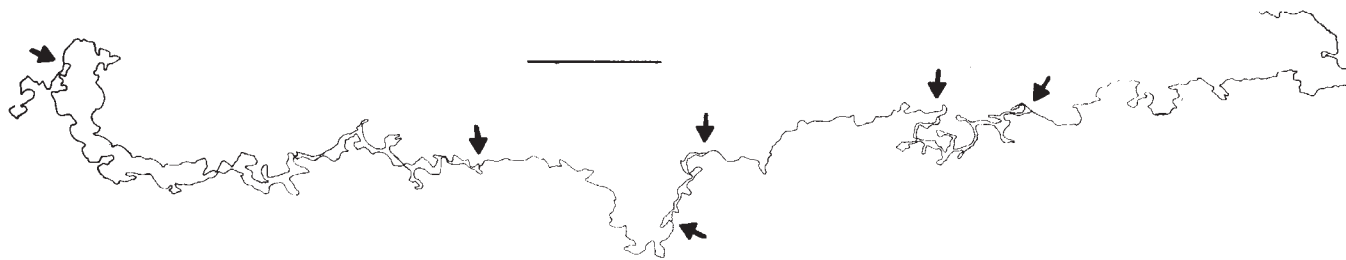


Fig. 4 Tracing of an electron micrograph of a replicating molecule. DNA was prepared and photographed as described in Fig. 2. The molecule shown is 117 μm long and the bubbles (right to left) are 13, 6, and 35 μm . Branch points are indicated by arrows. The bar represents 5 μm .

shift, samples were taken from the culture maintained at 38° C and the culture which had been shifted to 23° C (arrows, Fig. 3) and DNA was prepared for electron microscopy.

In the DNA from the sample collected at 38° C, 149 out of 150 molecules examined were simple linear structures. In the sample collected 20 min after the shift to 23° C, 66% (thirty-nine out of fifty-nine) of the DNA molecules observed contained bubbles or forks of the kind found in the density experiments. The correlation of such molecules, which are not present in the mutant at the restrictive temperature, with the burst of DNA synthesis, as measured by incorporation of radioactive label, is again consistent with the hypothesis that these molecules are replication intermediates. The total amount of DNA replicated in this sample can be estimated from the ratio of the sum of lengths of one arm of all bubbles and forks to the total length of all molecules measured. This ratio is 0.1 and is consistent with the incorporation data (Fig. 3), indicating that about 6% of a round of replication has occurred at the 20 min time point. The replicating molecules isolated from the cells in S phase varied in size from 47 to 250 μm . Thus, they probably represent intact chromosomal DNAs³, and it is unlikely that extensive double-strand breakage occurs during the isolation of the molecules or during yeast replication.

From analysis of the data obtained in the synchronous culture experiment, a number of general conclusions can be made about yeast chromosomal DNA replication. The appearance of bubbles is most easily explained by internal initiation. Since most (thirty-four out of thirty-nine) of the replicating molecules contained bubbles, it is likely that most yeast DNA molecules initiate synthesis internally. The presence of Y-shaped molecules suggests either that there are some initiation sites at the ends of the chromosomes, or that internal initiations occur sufficiently close to the ends of chromosomes that, after a small amount of DNA synthesis, a Y-shaped inter-

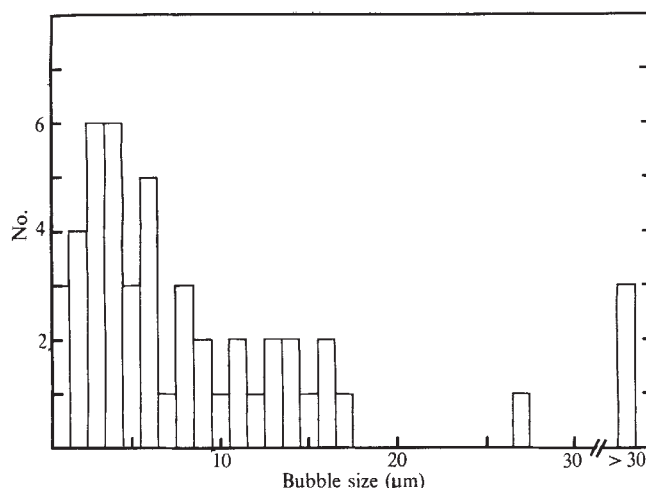


Fig. 6 Distribution of bubble size. Measurements of the size of bubbles in replicating molecules from the experiment in Fig. 3 were made on photographs at a magnification of 10,000 to 23,000.

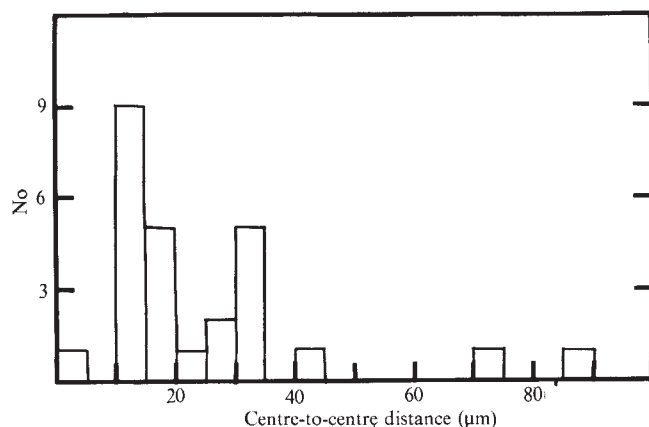


Fig. 5 Distribution of centre-to-centre distances between bubbles in molecules containing more than one bubble. Measurements of the centre-to-centre distances between bubbles in the replicating molecules from the experiment in Fig. 3 were made with a Keuffel and Esser map measurer on photographs at a magnification of 10,000.

mediate is formed. Bacteriophage T7 DNA has a Y-shaped intermediate throughout most of its replication although it initiates internally⁷.

At least some yeast chromosomes contain multiple initiation sites. In the S phase sample, 31% (twelve out of thirty-nine) of the replicating molecules contained more than one bubble. Figure 4 shows a tracing of a molecule with three bubbles. The size of replication units can be estimated from the spacing of bubbles along molecules. If a replication unit is defined as the amount of DNA replicated from a single initiation site, then a measure of the centre-to-centre distance between bubbles provides an estimate of this size¹⁴. The centre-to-centre distance between bubbles varied from 3 μm to 86 μm . Figure 5 shows that the distribution is not random; most of the distances fall into two clusters, one at 15 to 20 μm and the other at 30 to 35 μm . These values represent upper limits for replication unit sizes since some units may not have been activated in the observed molecules and some adjacent units may have already fused. Most bubbles are smaller than the smallest replication unit (Figs 5 and 6), so it is unlikely that many bubbles represent fused units. Thus it seems that replication unit size in yeast is more like that of higher eukaryotes^{14,15} than that of prokaryotes^{16,17}.

The replication units on a chromosomal DNA molecule seem to initiate replication at different times. If all units initiated at the same time, then all bubbles on a molecule should be the same size (assuming that the rate of replication at all forks is constant); however, we find bubbles of varying sizes on individual molecules (for example, Fig. 4). The data also indicate that many yeast chromosomes in the synchronised cells initiate replication early in the S phase. This is shown by the fact that 65% of the molecules contained replication structures at a time when only about 10% of the DNA had replicated. Thus, at least 65% of yeast chromosomes initiate

within the first 10% of S. This estimate could be in error for two reasons. Any asynchrony in the initiation of DNA synthesis among cells in the population would lead to an underestimate of the number of molecules which initiate synthesis at the beginning of S. In addition, mechanical shear during spreading of DNA could alter the apparent percentage of replicating molecules.

In summary, we find that yeast DNA replication intermediates include bubble-containing structures and Y-shaped structures. DNA molecules containing these structures are found in cultures in S phase, but not in cultures blocked before the initiation of DNA synthesis. In addition, there is at least a thirty-fold enrichment for molecules containing these structures in the region of a CsCl gradient where partially replicated molecules are expected to band. The same kinds of structures have been observed in DNA from asynchronous cultures³. Therefore, these structures cannot be attributed to abnormal growth conditions induced by density labelling or to the synchronisation procedure.

DNA replication in yeast is similar to replication in higher eukaryotes in several ways. Most, if not all, initiation sites are internal. Many chromosomes contain more than one replication unit, and the size of replication units is similar to that in higher eukaryotes. Because we can observe molecules which are almost certainly intact chromosomal DNAs, however, experiments which are very difficult to do in higher eukaryotes are possible. For example, replication maps of individual chromosomes can be made which include the location of initiation sites and the sequence of replication of different units.

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- ¹ Hartwell, L. H., *A. Rev. Genet.*, **4**, 373 (1970).
- ² Petes, T. D., and Fangman, W. L., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1188 (1972).
- ³ Petes, T. D., Byers, B., and Fangman, W. L., *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- ⁴ Ogawa, T., Tomizawa, J.-I., and Fuke, M., *Proc. natn. Acad. Sci. U.S.A.*, **60**, 861 (1968).
- ⁵ Schnös, M., and Inman, R. B., *J. molec. Biol.*, **51**, 61 (1970).
- ⁶ Delius, H., Howe, C., and Kozinski, A. W., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 3049 (1971).
- ⁷ Dressler, D., Wolfson, J., and Magazin, M., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2682 (1972).
- ⁸ Davis, R. W., Simon, M., and Davidson, N., *Methods in Enzymology*, **21**, 413 (1971).
- ⁹ Lang, D., *J. molec. Biol.*, **54**, 557 (1970).
- ¹⁰ Inman, R. B., and Schnös, M., *J. molec. Biol.*, **56**, 319 (1971).
- ¹¹ Wolfson, J., and Dressler, D., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2682 (1972).
- ¹² Bücking-Throm, E., Duntze, W., Hartwell, L. H., and Manney, T. R., *Expl. Cell Res.*, **73**, 99 (1973).
- ¹³ Hartwell, L. H., *J. Bact.*, **104**, 1280 (1970).
- ¹⁴ Huberman, J. A., and Riggs, A. D., *J. molec. Biol.*, **32**, 327 (1968).
- ¹⁵ Callan, H. G., *Proc. R. Soc. London, B*, **181**, 19 (1972).
- ¹⁶ Cairns, J., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 43 (1963).
- ¹⁷ Bode, H. R., and Morowitz, H. J., *J. molec. Biol.*, **23**, 191 (1967).

Preferential DNA Repair in Human Cells

EXCISION repair plays a vital role in the recovery of human cells from ultraviolet irradiation¹, but it does not remove all lesions from DNA, even when they are as potent as cyclobutane pyrimidine dimers. In fact, only about 50% to 75% of the dimers produced by low fluences of ultraviolet light are excised and the remainder persist in the DNA for at least 24 to 48 h²⁻⁴. We have investigated why some but not all dimers are excised. Our conclusion is that dimers, and possibly other ultraviolet photoproducts, persist in tracts of DNA which are rendered refractory to excision repair by a 'mask' of protein.

Human fibroblasts (WI-38) were grown in culture, labelled with ³H-thymidine, irradiated with ultraviolet light, incubated for various periods and then enzymatically assayed⁵ for DNA damage by using a *Micrococcus luteus* extract containing repair endonucleases (see legend to Fig. 1). This assay mimics the first step of *in vivo* excision repair, namely the endonucleolytic nicking of DNA in the vicinity of pyrimidine dimers, so we expected that, if nucleoproteins did indeed interfere with excision repair *in vivo*, they would likewise interfere with the *in vitro* assay for DNA damage. To test this hypothesis the cellular DNA was assayed for damage after exposure to either low (less than 0.15 M) or high (2 M NaCl) concentrations of salt, that is, conditions which ensured that histones, and possibly other proteins, either remained bound to, or were stripped from⁶, the chromosomal DNA. We did in fact find that exposing the chromosomal DNA to a high concentration of salt greatly increased the

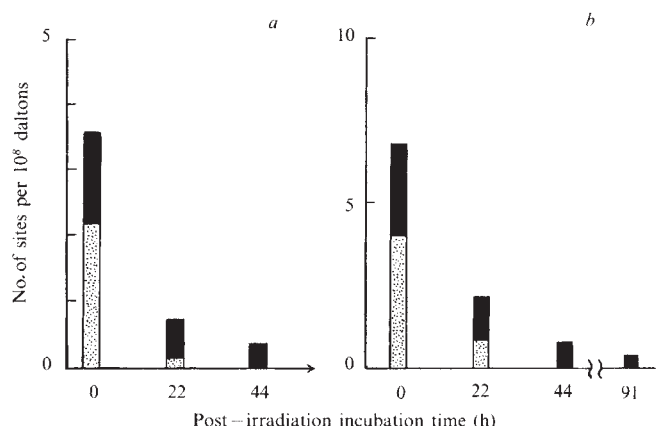


Fig. 1 Disappearance of ultraviolet-induced endonuclease sensitive sites from the DNA of human fibroblasts after 50 (a) or 100 (b) erg mm⁻² of 254 nm ultraviolet light. The sites exposed under low salt conditions (dotted) and the additional sites exposed by high salt (black) were assayed essentially by the method described elsewhere⁵. Approximately 100,000 cells (WI-38 originally derived from embryonic lung tissue) were plated into 60-mm plastic dishes and incubated overnight in Eagle's medium with 10% dialysed foetal calf serum and 1 μ Ci of ³H-thymidine added to label the DNA. After ultraviolet irradiation, cells were re-incubated for various times and then harvested by scraping into ice-cold saline-EDTA solution. For the endonuclease assay the cells were first rendered porous by osmotic shock, that is, they were concentrated to 5×10^6 cells ml⁻¹ in 10% sucrose, then diluted six-fold in water and vortexed. The sample was then divided into two, one of which was made 2 M with respect to NaCl and kept at 0° C for 5 min. Both samples were then diluted a further ten-fold with water and then incubated with *M. luteus* extract for 12 min at 37° C. Fifty microlitres of each sample was then layered on an alkaline sucrose gradient so that the cellular DNA content could be sedimented through alkali to detect single-strand breaks produced by endonuclease action, that is, endonuclease-sensitive sites⁵. In control experiments using either unirradiated cells or no *M. luteus* extract, the weight average molecular weight of the DNA was always in the range of 0.9 to 1.1×10^8 daltons, so we normalised all our results to the number of endonuclease-sensitive sites per 10^8 daltons of DNA. These values are accurate to within ± 0.1 site.

yield of detectable endonuclease-sensitive sites (Fig. 1). In cells which were assayed immediately after ultraviolet irradiation and had, therefore, no chance to repair DNA damage, over 60% more sites were revealed by the high salt assay. In other words, at least 40% of the chromosomal DNA is normally inaccessible to exogenous repair endonucleases used in the *in vitro* assay. This is a conservative estimate because some reassociation of protein and DNA probably occurs when the 2 M NaCl is diluted ten-fold to perform the assay. We do not believe, however, that much more than 50% of the DNA is inaccessible because in earlier experiments⁶ employing low salt concentrations it was estimated that about half of the dimers induced in the DNA did give rise to detectable endonuclease sites.

The sites which are normally inaccessible to the exogenous repair endonucleases also seem to be relatively inaccessible to the DNA repair systems which act *in vivo*. Thus, while all of the ultraviolet-induced sites initially detectable under low salt assay conditions are removed from the DNA of cells during 44 h of post-irradiation incubation (Fig. 1 and ref. 5), many of the additional sites exposed by the high salt assay still persist in the DNA. For instance, 44 h after exposure to a fluence of 100 erg mm⁻² of ultraviolet light, 28% of these additional sites still remain in the DNA and, even after 91 h, 13% still persist (Fig. 1).

The simplest explanation for our results is that some tracts of DNA are masked from the action of repair enzymes by sheaths of nucleoprotein. *In vitro* this protein can be removed from the DNA by high salt concentrations while *in vivo* it seems that repair systems can slowly gain access to the DNA, possibly because of changes that occur in DNA-protein complexes during DNA replication, or cell division, or both. We estimate that about 40% of the DNA is masked in this way and that the tracts covered with protein are no more than 10⁶ daltons long because when using the low salt assay we did not observe any higher molecular weight DNA which was free of endonuclease-sensitive sites. Such conclusions are reasonable because it is known that about half of the DNA in the chromatin of mammalian cells is 'open' and linked together by many short tracts of DNA covered with protein⁶.

Two general comments on our results are appropriate. First, it is possible that damage induced by agents other than ultraviolet light could also persist in these masked tracts of DNA and, as mammals age, their cells might accumulate such damage, especially in the absence of cell division. Second, differentiated cells might tolerate considerable damage in these masked, and possibly genetically unexpressed⁷, portions of DNA. Thus the de- or re-differentiation of a cell might have a lethal, mutagenic or possibly carcinogenic⁸ consequence if expression of this DNA occurred before repair could take place.

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Note added in proof. Recent work^{9,10} using a similar technique also indicates that there are both fast and slow components to the repair system which removes ultraviolet-induced endonuclease sensitive sites from the DNA of human cells.

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- ¹ Cleaver, J. E., in *Nucleic Acid-Protein Interactions, Nucleic Acid Synthesis in Viral Infection*, Miami Winter Symposium (edit. by Ribbons, D. W., Woessner, J. F., and Schultz, J.), 2, 87 (North-Holland, Amsterdam, 1971).
- ² Regan, J. D., Trosko, J. E., and Carrier, W. L., *Biophys. J.*, **8**, 319 (1968).
- ³ Setlow, R. B., Regan, J. D., German, J., and Carrier, W. L., *Proc. nat. Acad. Sci. U.S.A.*, **64**, 1035 (1969).
- ⁴ Cleaver, J. E., and Trosko, J. E., *Photochem. Photobiol.*, **11**, 547 (1970).
- ⁵ Wilkins, R. J., *Int. J. Radiat. Biol.* (in the press).
- ⁶ Clark, R. J., and Felsenfeld, G., *Nature new Biol.*, **229**, 101 (1971).
- ⁷ DeLange, R. J., and Smith, E. L., *A. Rev. Biochem.*, **40**, 279 (1971).
- ⁸ Markert, C. L., *Cancer Res.*, **28**, 1908 (1968).
- ⁹ Paterson, M. C., Lohman, P. H. M., and Sluyter, M. L., *Mutation Res.*, **19**, 245 (1973).
- ¹⁰ Paterson, M. C., Lohman, P. H. M., Westerveld, A., and Sluyter, M. L., *Nature* (in the press).

Histone mRNA and Histone Synthesis during Embryogenesis

PROPORTIONAL contributions of individual histone fractions to total histone and their degree of microheterogeneity change during development. This has now been established for many plants and animals^{1,2} including sea urchins³⁻⁵. The very lysine-rich histones, designated F1, can be fractionated as several proteins of similar amino acid composition^{7,9} and as different phosphorylated derivatives^{10,11}. These histones in particular show considerable species specificity in primary structure, molecular weight, and microheterogeneity^{8,9,12,13}. It has been demonstrated in earlier studies³⁻⁶ that aside from changes in proportion among histone classes synthesised during sea urchin development, synthesis of an F1 characteristic of the morula stage (F1-m) gives way to synthesis of a new and electrophoretically distinct F1 at the gastrula stage (F1-g). Significantly, the F1-m histone is neither degraded nor converted to F1-g during the interval from morula to gastrula⁶.

We report here experiments designed to distinguish between two alternative modes of control over these developmentally programmed changes: differences in the template RNA from which histones are translated and

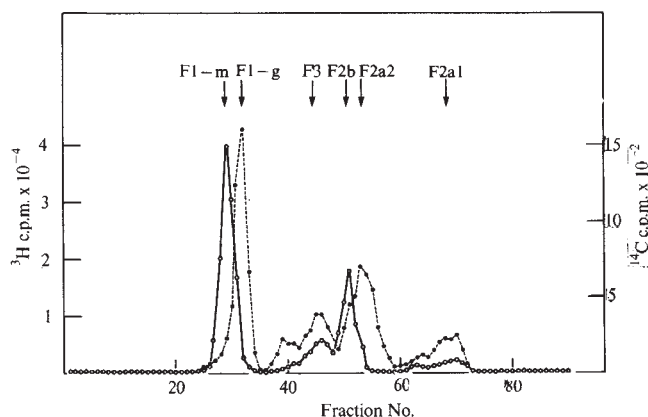


Fig. 1 Acetic acid-urea acrylamide gel electrophoresis of *L. pictus* embryo histones labelled *in vivo*. Two millilitres of morulae (approximately 2×10^6 embryos at about 100 cells) was incubated in 100 ml sterile seawater with 200 μ Ci ³H-lysine (20.8 Ci mmol⁻¹) for 1 h. Two millilitres of gastrulae (0.5×10^3 to 1.0×10^3 cells) was incubated in a volume of 100 ml with 20 μ Ci ¹⁴C-lysine (260 mCi mmol⁻¹) for 2 h. Histones were isolated²¹ and dissolved in 2.5 M urea, 0.9 N acetic acid, 0.2% 2-mercaptoethanol. Electrophoresis was at 2 mA per gel for 5-6 h on 10% acrylamide, 2.5 M urea, pH 2.7 acetic acid gels, 14 cm long¹⁸. The histone-containing portion of the gel was sliced and hydrolysed for counting. ○—○, ³H-lysine morula histones; ●---●, ¹⁴C-lysine gastrula histones.

differences in the way histones from an invariant soluble pool associate with DNA in the chromatin. The experiments are designed also to distinguish between primary structure differences and differential modification after synthesis as the chemical basis for the different electrophoretic mobilities of F1-m and F1-g.

Newly synthesised histones of *Lytechinus pictus* morulae and gastrulae were first examined by acetic acid-urea gel electrophoresis of the proteins labelled in a pulse exposure to ^3H -lysine¹⁴. The results are shown in Fig. 1. Morulae synthesise a lysine-rich histone (F1-m) that migrates more slowly in this electrophoretic system than does the F1 made by gastrulae (F1-g). Proportional differences of incorporation among the other four histone classes, particularly in the ratio F2b/F2a2, were observed as usual⁶.

Morula and gastrula histones were then separated by electrophoresis on sodium dodecyl sulphate (SDS) gels, according to the method of Laemmle¹⁵. This analysis shows (Fig. 2) that morulae synthesise only F1-m, with molecular weight $\approx 20,000$. First observed between hatching and gastrulation is the synthesis of F1-g (molecular weight $\approx 21,000$). By the time gastrulation is in progress, incorporation of tracer amino acid into F1-m is greatly reduced and labelled F1-g accounts for most of the new F1. Electrophoretic mobilities of F1-m and F1-g are not altered detectably in either separation system after treatment with alkaline phosphatase (data not shown here). Seale and Aronson⁴ report similar findings in experiments with another species, *Strongylocentrotus purpuratus*.

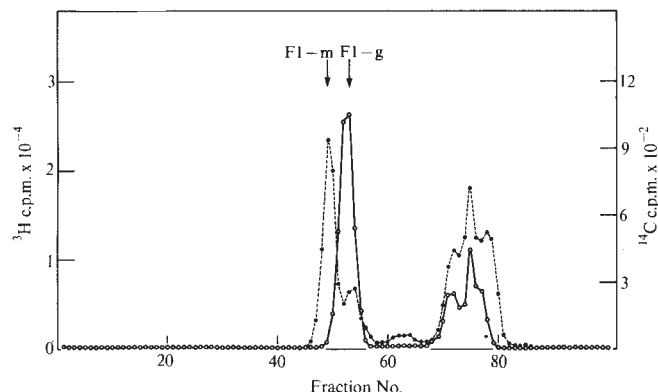


Fig. 2 SDS acrylamide gel electrophoresis of morula and gastrula histones. Histones, prepared as described under Fig. 1, were dialysed against 0.1% SDS, 0.2% 2-mercaptoethanol, and separated on 12.5% acrylamide-SDS gels¹⁹. ○—○, ^3H -lysine morula histones; ●—●, ^{14}C -lysine gastrula histones.

Total polyribosomal RNA was next prepared, purified, and translated in the ascites cell-free protein synthesis system employed by Gross *et al.*¹⁶ for earlier studies on histone mRNA. Protein products of cell-free synthesis (Table 1), together with marker histones synthesised *in vivo* at the gastrula stage, were subjected to co-electrophoresis on acetic acid-urea gels. As is shown in Fig. 3a, RNA from morula polyribosomes directs the synthesis of F1-m. Total histones synthesised *in vitro* under direction of this RNA also give a high F2b/F2a2 incorporation ratio, which is characteristic of labelled histone that associates with chromatin *in vivo* at this stage of development. Gastrula polyribosomal RNA, by contrast, directs the cell-free synthesis of F1-g (Fig. 3b). The gastrula RNA-directed histones have the low F2b/F2a2 incorporation ratio characteristic of protein synthesis in the living embryo at this stage. In electrophoresis on SDS gels (Fig. 4), protein products made *in vitro* under the direction of morula RNA are again observed to contain only F1-m, while poly-

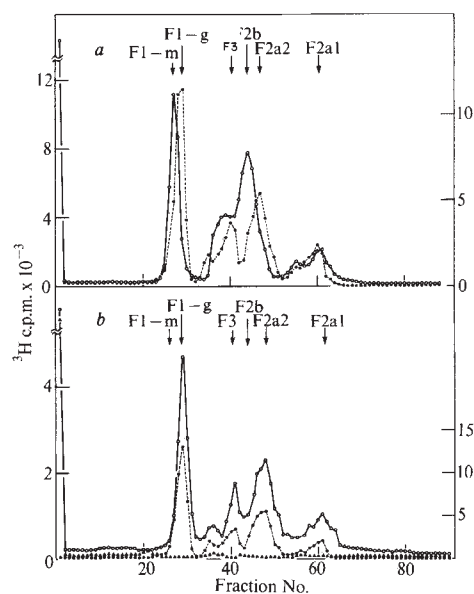


Fig. 3 Electrophoresis of products of cell-free protein synthesis on pH 2.7 acrylamide gels. 46 μl of the reaction mixture described in Table 1 was treated with DNase I (50 $\mu\text{g ml}^{-1}$) at 30°C for 20 min and then dialysed against 2.5 M urea, 0.9 N acetic acid, 0.2% 2-mercaptoethanol at 4°C. ^{14}C -lysine-labelled gastrula histones (*in vivo* marker) were added to the dialysed sample. The mixture was separated as described under Fig. 1. Only the histone-containing region is shown. a: ○—○, cell-free products directed by morula RNA; ●—●, product of synthesis *in vivo* at gastrula stage. b: ○—○, cell-free products directed by gastrula RNA; ●—●, gastrula marker histones; ▲—▲, cell-free products, no added RNA.

ribosomal RNA from gastrulae yields products that separate on these gels as F1-g and a small amount of F1-m.

The postribosomal supernatant fraction of these embryos contains RNA with template activity in the cell-free system. Morula RNA of this kind directs the morula-specific pattern of lysine incorporation; little or no F1-g is labelled. Gastrula supernatant RNA contains sequences that encode and direct the synthesis of F1-g (data not shown here).

Polyribosomal (messenger) RNA from two different stages of development therefore contains sequences that direct histone synthesis *in vitro* according to patterns that match qualitatively, and to a considerable extent quantitatively, the corresponding patterns of synthesis *in vivo*. Mobility differences between the very lysine-rich histones encoded on and directed by the two kinds of template RNA are

Table 1 Cell-free Lysine Incorporation into Proteins Directed by Morula and Gastrula Polyribosomal RNA

Addition	^3H -lysine incorporated (c.p.m.)	Stimulation (X blank)
Blank (no RNA)	495	—
Morula RNA	6,126	12.4
Gastrula RNA	3,612	7.3

Polyribosomal RNA was prepared from 6 ml packed morulae and 8 ml gastrulae. Embryos were washed twice with Ca^{2+} - Mg^{2+} -free seawater, once with TNM (0.2 M NaCl, 5 mM Mg acetate, 10 mM Tris, pH 7.5), and homogenised in 2.5 volumes of TNM. The 12,000g supernatant was centrifuged through a 7–47% sucrose gradient for 3 h at 26,000 r.p.m. in a Spinco SW27 rotor. Polyribosomes were collected from appropriate fractions and their RNA extracted. This RNA was translated in the Krebs II ascites-derived cell-free system^{16,22,23}. Input RNA was at 22.5 μg , prepared from morulae and gastrulae. The incubation system contained 10 μC ^3H -lysine at 20.8 Ci mmol⁻¹, plus 50 μM unlabelled amino acids (less lysine), in a volume of 50 μl . Incubation was for 60 min at 30°C. Two aliquots of 2 μl from each incubation were precipitated with hot 5% trichloroacetic acid and the precipitated radioactivity counted.

very likely to be due to differences in primary structure of the two proteins. The differences can in any case not be due to differential post-translational modification or derivatisation, since the information content of the two mRNA preparations is expressed through a constant enzymatic machinery, the components of the ascites cell-free incorporation system.

We conclude, therefore, that histones F1-m and F1-g of morulae and gastrulae, respectively, co-migrating as they do with corresponding products of cell-free synthesis directed by morula and gastrula RNA, also differ in their primary structures.

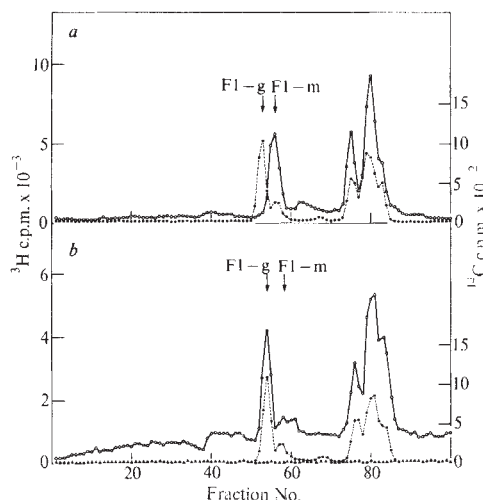


Fig. 4 Analysis of cell-free products on SDS-acrylamide gels. Morula or gastrula RNA was incubated with the cell-free protein synthetic system as described, and co-electrophoresis with ^{14}C -lysine gastrula marker histones was carried out as in the experiments of Fig. 2. *a*: ○—○, morula RNA-directed cell-free products; ●—●, gastrula marker histones. *b*: ○—○, gastrula RNA-directed cell-free products; ●—●, gastrula marker histones; ▲—▲, cell-free products, no added RNA.

Although other cases of histone tissue specificity are known^{22,23}, histone F1-g, which becomes detectable only after hatching in *Lytechinus* and other sea urchins^{4,6}, may be an example (so far rare) of a qualitatively new histone appearing in the course of early development. It is possible, of course, that traces of F1-g are present in pre-hatching stages and are not detected by methods such as were employed here, but this hardly affects the argument. Quantitative changes in the fractional synthesis of histones other than the F1 appear also to be regulated, at least in part, by the relative amounts of their corresponding messenger RNA in polyribosomes.

The situation in *Arbacia punctulata*, another species studied by the methods described above, is in essence the same as in *Lytechinus*, but with some minor modifications. Mobility relations between the two *Arbacia* F1 fractions differ from those of *Lytechinus* when the proteins are separated on acetic acid-urea gels. SDS gel electrophoresis reveals that the switch-over occurs earlier than in *Lytechinus*, and it seems to be completed by the late gastrula stage.

The data indicate, in summary, that the kinds and amounts of histones that associate with DNA in chromatin do in fact differ characteristically from one stage of development to the next and that the differences are in large part the result of differential messenger content of the translational machinery.

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- ¹ Allfrey, V. G., in *Histones and Nucleohistones* (edit. by Phillips, D. M. P.) (Plenum Press, New York, 1971).
- ² Elgin, S. C. R., Froehner, S. C., Smart, J. E., and Bonner, J., *Adv. cell molec. Biol.*, **1**, 1 (1971).
- ³ Easton, D., and Chalkley, R., *Expl. Cell Res.*, **72**, 502 (1972).
- ⁴ Seale, R., and Aronson, A., *J. molec. Biol.*, **75**, 647 (1973).
- ⁵ Johnson, A. W., and Hnilica, L. S., *Biochim. biophys. Acta*, **246**, 141 (1971).
- ⁶ Ruderman, J. V., and Gross, P. R., *Devl Biol.* (in the press).
- ⁷ Kincaid, J. M., and Cole, R. D., *J. biol. Chem.*, **241**, 5798 (1966).
- ⁸ Bustin, M., and Cole, R. D., *J. biol. Chem.*, **243**, 4500 (1968).
- ⁹ Kincaid, J. M., *J. biol. Chem.*, **244**, 3375 (1969).
- ¹⁰ Sherod, D., Johnson, G., and Chalkley, R., *Biochemistry*, **9**, 4611 (1970).
- ¹¹ Langan, T., *Proc. natn. Acad. Sci. U.S.A.*, **64**, 1276 (1969).
- ¹² Panyim, S., and Chalkley, R., *J. biol. Chem.*, **246**, 7557 (1971).
- ¹³ Panyim, S., and Chalkley, R., *J. biol. Chem.*, **246**, 4206 (1971).
- ¹⁴ Panyim, S., and Chalkley, R., *Archs Biochem. Biophys.*, **130**, 137 (1969).
- ¹⁵ Laemmle, U. K., *Nature*, **227**, 280 (1970).
- ¹⁶ Gross, K. W., Jacobs-Lorena, M., Baglioni, C., and Gross, P. R., *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- ¹⁷ Panyim, S., and Chalkley, R., *Biochem. Biophys. Res. Commun.*, **37**, 1042 (1969).
- ¹⁸ Sheridan, W. F., and Stern, H., *Expl. Cell Res.*, **45**, 323 (1967).
- ¹⁹ Neelin, J. M., Callahan, P. X., Lamb, D. C., and Murray, K., *Can. J. Biochem. Physiol.*, **42**, 1743 (1964).
- ²⁰ Appels, R., Well, J. R., and Williams, A. F., *J. Cell Sci.*, **10**, 47 (1971).
- ²¹ Bontinen, L. C., and Comb, D. G., *J. molec. Biol.*, **57**, 355 (1971).
- ²² Hogan, B., and Gross, P. R., *J. cell Biol.*, **49**, 692 (1971).
- ²³ Gross, K. W., Ruderman, J. V., Jacobs-Lorena, M., Baglioni, C., and Gross, P. R., *Nature new Biol.*, **241**, 272 (1973).

Geographical Distribution of Australia Antigen Determinants d, y and w

AUSTRALIA antigen (Au) has a group of antigenic determinants on its surface, which may occur in different combinations in the sera of people living in different geographical locations. Several of these determinants have been characterised and given alphabetical designations¹⁻⁵. It has been reported that determinants d and y tend to be mutually exclusive⁶ although rarely they occur together (S. M. and S. B., unpublished). Determinants w and r also tend to occur in different samples rather than together and these two determinants have a strongly marked geographical distribution. Determinant w is common in the USA, South America, Europe and Africa, while r is common in Asia and the Pacific area^{5,7}. We have tested 893 samples of Australia antigen from locations around the world for determinants d, y and w. Here we present these findings and speculate on factors which may contribute to the geographical distribution of Australia antigen subtypes.

The Australia antigen samples included in this study were all from apparently healthy asymptomatic carriers. They are part of the blood collection of the Division of Clinical Research at the Institute for Cancer Research and had previously been found to contain Australia antigen by immunodiffusion testing. Australia antigen determinants were identi-

Table 1 Distribution of w Determinant with Respect to Subtypes d, y and a in Asymptomatic Carriers from Various Geographical Locations

	N	D		Y		A	
		w ⁺	w ⁻	w ⁺	w ⁻	w ⁺	w ⁻
Northern Europe							
Belgium	2	2	0	0	0	0	0
Finland	31	31	0	0	0	0	0
Germany	4	2	0	2	0	0	0
Total	37	35	0	2	0	0	0
Mediterranean							
Turin, Italy	17	4	0	13	0	0	0
Yugoslavia	9	4	0	5	0	0	0
Total	26	8	0	18	0	0	0
Africa							
Ghana	6	0	0	6	0	0	0
Ivory Coast	3	0	0	3	0	0	0
Nigeria	5	0	0	5	0	0	0
South Africa (Africans)	2	1	0	1	0	0	0
Senegal	14	0	0	14	0	0	0
Total	30	1	0	29	0	0	0
Asia							
China	4	3	1	0	0	0	0
Japan	91	25	66	0	0	0	0
Philippines	5	5	0	0	0	0	0
Philippines (Cebu)	42	38	2	2	0	0	0
Thailand	3	2	1	0	0	0	0
Total	145	73	70	2	0	0	0
Far East							
India	20	2	0	16	1	0	1
Australia							
Aborigine	14	0	0	12	0	2	0
Oceania							
American Samoa	3	0	2	1	0	0	0
Bougainville							
North	34	0	34	0	0	0	0
South Central	172	17	75	42	35	1	2
Tairara	5	0	5	0	0	0	0
Total	211	17	114	42	35	1	2
French Polynesia							
Bora Bora	3	2	1	0	0	0	0
Tahiti	13	6	7	0	0	0	0
Tubai	1	0	0	0	0	1	0
Total	17	8	8	0	0	1	0
Malaita	63	2	61	0	0	0	0
New Caledonia							
Konmoi	2	0	1	1	0	0	0
La Foa	6	0	5	1	0	0	0
Noumea	5	3	1	1	0	0	0
Total	13	3	7	3	0	0	0
New Guinea							
Various groups	14	10	4	0	0	0	0
New Hebrides							
Aoba	8	0	8	0	0	0	0
Lamap	16	1	15	0	0	0	0
Norsup	1	1	0	0	0	0	0
Tanna	17	0	9	0	8	0	0
Vate	27	1	26	0	0	0	0
Total	69	3	58	0	8	0	0
Ponape	5	0	5	0	0	0	0
Rongelap	6	1	5	0	0	0	0
Uitirik	7	0	7	0	0	0	0
Central and South America							
Brazil	3	1	0	2	0	0	0
Dominican Republic	19	11	0	8	0	0	0
Jamaica	2	0	0	2	0	0	0
Peru (Cashinahua)	35	33	1	0	0	1	0
Surinam	11	8	0	3	0	0	0
Yucatan	3	3	0	0	0	0	0
Total	73	56	1	15	0	1	0
North America							
Frobisher Bay, Canada	2	2	0	0	0	0	0
Philadelphia	22	18	0	4	0	0	0
Philadelphia (prisoners)	26	18	0	8	0	0	0
Red Cross donors	90	76	1	13	0	0	0
Total	140	114	1	25	0	0	0

fied using the standard seven hole immunodiffusion pattern cut in 1.1% agarose in pH 8.2 veronal buffer⁸. Specific typing sera were put in the centre well, control Australia antigen was put in the top and bottom wells, and test samples in the remaining four wells. Human anti-aw was reacted against an aw control antigen adjacent to the test samples. The formation of a line of identity between the control antigen and the test antigen was interpreted as indicating that the test antigen was w⁺. When a spur was formed by the control antigen over the test antigen it was considered w⁻. The same principle was used in testing for determinant d with guinea pig anti-ad antiserum (NIH V 801-502-058). A few sera which were known to be Australia antigen positive did not react with this serum at all. These will be considered in detail elsewhere, but here they were considered d⁻. These and all other d⁻ sera were retested with a second anti-ad serum for confirmation of their d⁻ status. Absorbed chimpanzee anti-y and y control antigen were used to test for determinant y. Sera which were negative for both d and y were tested with an additional anti-ay serum.

The antigens have been grouped according to geographical origin of the sera and have been assigned to subtype categories suggested by Le Bouvier⁶ according to the presence of determinants d and y (Table 1). Subtype A consists of those antigens which lack both the d and y determinants. This group is designated A because by definition it must have determinant a which is defined as the universal or group determinant. The subtype categories have been further divided into w⁺ and w⁻. In all but four cases, determinants d and y were found to be mutually exclusive. These are not shown in Table 1. Two of these four exceptions were found on Bougainville and the other two were from people living in the United States who had travelled in Asia.

Australia antigen with determinant d is common in asymptomatic carriers in the United States, North Europe, Asia and Oceania, although subtype y does occur in these areas. All antigen samples from Japan carry the d determinant while none carry y. Antigen with the y determinant occurs almost to the exclusion of d in Africa and in Australian aborigines. Determinant y occurs frequently in India and in the Mediterranean area in Italy and Yugoslavia.

Virtually all antigens from Europe, the United States, Africa, India and Australia carry the w determinant. Antigen without w predominates in the New Hebrides, Micronesia, New Caledonia, Bougainville, Japan, Malaita and Thailand (Table 2). Both w⁺ and w⁻ occur in the population in China, the Philippines, French Polynesia, and New Guinea.

From these data and the data in Table 2, it seems that there are marked geographical separations of subtype. The basic northern European subtype is adw⁺ y⁻ and the African subtype is ayw⁺ d⁻. In the Mediterranean area these subtypes both occur concurrently. The Asiatic type seems to be mostly adw⁺ y⁻ with some ad⁺ wy⁻. Large areas of

Table 2 Distribution of Subtypes in Asymptomatic Persons Previously Described

Location	Subtype D	Subtype Y
Tonga ⁹	30	11
Thailand ⁵	21 *	2
Korea ⁵	4 †	3
New Guinea ²⁰	9	0
Singapore (China) ²⁰	24	1
Sweden ²¹	16	3
Germany ‡	30	2
Denmark ²²	89	5
Israel§	13	47
Iran ²³	0	23

* 2 ayw, 20 adr; 1 probably adw.

† 3 ayw 4 adr.

‡ A. Schober and R. Thomssen, personal communication.

§ S. Bar-Shany, V. M. Edwards, J. W. Mosley, and W. H. Bancroft, personal communication.

Oceania are $ad^+ wy^-$. Japan is at a geographical interface with both $adw^+ y^-$ and $ad^+ wy^-$. India and Australian aborigines are mostly $ayw^+ d^-$ and Melanesia is in the interface between Australia ($ayw^+ d^-$) and the rest of Oceania ($ad^+ wy^-$).

Figure 1 shows the distribution of dominant subtypes from different parts of the world tested in this study.

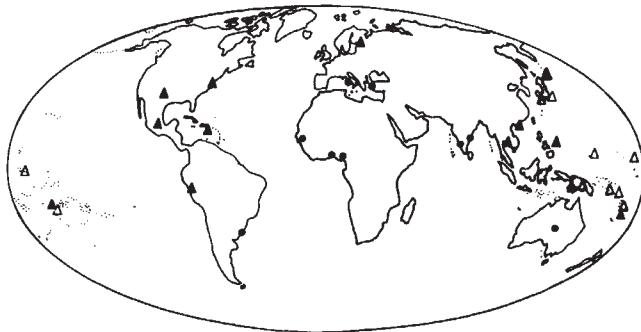


Fig. 1 Distribution of Australia antigen subtypes. Each symbol represents the dominant subtype in that location. Triangles represent subtype d. Circles represent subtype y. Solid figures represent w^+ ; open figures w^- .

One hypothesis generated by this study is that the Australia antigen subtypes among asymptomatic carriers could be used as population markers and serve as an indication of human migrations. This would be more likely if women were included in the migration since when women carry the antigen they can transmit it to some of their children who may then become chronic asymptomatic carriers⁹⁻¹⁴. When infants acquire Au from their mothers the subtype seems to be the same in both the mother and child^{14,15}. Daughters can then transmit the same subtype to their children and so on through many generations. This could maintain the subtype brought by the migrant population in a new environment. These introduced subtypes might not predominate since there are other mechanisms which produce carriers, but they should persist to some degree.

The subtype $ayw^+ d^-$ is most common in Africa. In all areas tested where African slaves were introduced the $ayw^+ d^-$ subtype occurs but in lower frequency than in Africa. These areas include the USA, Brazil, Jamaica, the Dominican Republic and Surinam. This is in contrast to northern Europe, Japan and Oceania where there are few people of African descent and little ay antigen. In the United States where about 10% (ref. 16) of the population is considered to be of African descent, 15% of the carriers are ay^{17} ; while in the Dominican Republic where 85% of the people have some African admixture¹⁸, 42% of the Australia antigen is $ayw^+ d^-$. This hypothesis could be further tested by correlating subtype data with other biological data (blood and serum groups) and linguistic, archaeological and cultural information concerning human migrations. An Au subtype marker would be unique among known biological markers since it behaves as a unit and is passed from mother to child unaltered. In contrast, inheritable human biological markers are subject to Mendelian segregation and the resulting phenotypes can change rapidly from generation to generation.

Two United States chronic carriers were found to have w^- antigen. They had both been in Vietnam and were presumed to have acquired their exotic subtype in Asia where w^- is more common. We have tested a total of 318 Au samples from both patients and asymptomatic carriers in the United States and all except those two have had the w determinant. It is probable that some veterans of the Pacific

theatre returned to the United States carrying $ad^+ wy^-$ at the end of the Second World War. This subtype has not spread in the United States population to any measurable extent. This is compatible with the assumption that maternal transmission is the major mechanism for preserving an introduced subtype in a new environment.

South Central Bougainville is unusual in that there are seven different Au subtypes. It is unlikely that this diversity could be due to chance. The diversity could be related to the fact that Bougainville is located between Australia where the subtype is entirely $ayw^+ d^-$ and Oceania where the complementary subtype $ad^+ wy^-$ occurs frequently. In areas such as Bougainville two subtypes could meet and recombine by a mechanism similar to crossover which may occur in a doubly infected host, either human, animal, or arthropod. For example, if $ayw^+ d^-$ and $ad^+ wy^-$ agents coexist in the same host it is possible that they could recombine and yield four types of Australia antigen; the original $ayw^+ d^-$ and $ad^+ wy^-$, as well as $ay^+ wd^-$ and $adw^+ y^-$ recombinations. All of these do in fact exist in Bougainville and have not been found together in other locations.

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- Levene, C., and Blumberg, B. S., *Nature*, **221**, 195 (1969).
- Raunio, V. K., London, W. T., Sutnick, A. I., Millman, L., and Blumberg, B. S., *Proc. Soc. exp. Biol. Med.*, **134**, 548 (1970).
- Le Bouvier, G. L., *J. infect. Dis.*, **123**, 671 (1971).
- Kim, C. Y., and Tilles, J. J., *J. infect. Dis.*, **123**, 618 (1971).
- Bancroft, W. H., Mundon, F. K., and Russell, P. K., *J. Immun.*, **109**, 842 (1972).
- Le Bouvier, G. L., McCollum, R. W., Hierholzer, jun., W. J., Irwin, G. R., Krugman, S., and Giles, J. P., *J. Am. med. Ass.*, **222**, 928 (1972).
- Mazzur, S., Falker, D., and Blumberg, B. S., *Nature new Biol.*, **243**, 44 (1973).
- Mazzur, S., *Am. J. med. Techn.*, **38**, 343 (1972).
- Turner, G. C., Field, A. M., Lasheen, R. M., Todd, R. M., White, G. B. B., and Porter, A. A., *Arch. Dis. Child.*, **46**, 616 (1971).
- Merrill, D. A., Dubois, R. S., and Kohler, P. F., *New Engl. J. Med.*, **287**, 1280 (1972).
- Okochi, K., Mayumi, M., and Yishioka, K., in *Proc. 3rd Int. Symp. Princess Takamatsu Cancer Research Fund*, Tokyo, November, 1972.
- Schweitzer, I. L., Wing, A., McPeak, C., and Spears, R. L., *J. Am. med. Ass.*, **220**, 1092 (1972).
- Aziz, M., Kahn, G., Khanum, T., and Siddiqui, A., *J. infect. Dis.*, **127**, 110 (1972).
- Mazzur, S., Blumberg, B. S., and Friedlaender, J. S., *Nature*, **247**, 41 (1974).
- Schweitzer, I. L., Edwards, V. M., Peters, R. L., and Mosley, J. W., *Gastroenterology*, **62**, 808 (1972).
- Encyclopedia Britannica*, **22**, 737 (1954).
- Dodd, R. Y., Holland, P. V., Ni, L. Y., Smith, H. M., and Greenwalt, T. J., *Am. J. Epid.*, **97**, 111 (1973).
- Department of State Publication 7759 (Dominican Republic, October 1970).
- Schmidt, N. J., Roberto, R. R., and Lennett, E. H., *Infect. Immun.*, **6**, 1 (1972).
- Simons, M. J., Binns, C. W., Malcolm, L. A., and Yap, E. H., *Papua New Guin. med. J.*, **15**, 91 (1972).
- Iwarson, S., Magnus, L., Lindholm, A., and Lundin, P., *Br. med. J.*, **1**, 84 (1973).
- Skinhøj, P., *Br. med. J.*, **1**, 418 (1973).
- Saidi, S., Farrohi, Kh., McCollum, R. W., and Le Bouvier, G. L., *Lancet*, **ii**, 1377 (1972).

Silent Maternal Transmission of Australia Antigen

CHRONIC asymptomatic carriers of Australia antigen (Au, hepatitis B antigen, HBAG, HAA and so on) are found in human populations throughout the world and they constitute an important reservoir for the maintenance of the infectious agent. Here we provide evidence which, in combination with previous data, suggests that an important mechanism for the establishment of these carriers is through the 'silent' (that is, without illness) transmission of chronic infections from generation to generation through the maternal line.

There have been several studies related to mothers with Australia antigen and their children. We analysed 109 completed or partially completed families, from five groups [Cebu, Bougainville, Lau and Baegu speaking areas of Malaita, and Sardinian¹ families from Turin]. There was a much higher frequency of Au in the offspring of matings in which the mothers were asymptomatic carriers of Au and the fathers were not, than in matings in which the fathers were carriers and the mothers were not (for all the families combined $P=0.55 \times 10^{-10}$ (ref. 2). Okochi and his colleagues found that of twelve children born to Japanese mothers who were asymptomatic carriers of Au, seven children became carriers within the first 6 months of life³. Papaevangelou found that of eleven asymptomatic Greek mothers who had Au, two had babies who developed the antigen at 6 and 14 months, respectively. The antigen did not persist in these infants (G. Papaevangelou, personal communication). Turner *et al.* made a careful documentation of transmission of antigen from a mother with hepatitis to her infant⁴. Subsequently, the infants of five mothers with Australia antigen positive hepatitis were studied and four were found to be carriers of the antigen without clinical signs of hepatitis⁵.

In a larger study of mothers living in California who had Australia antigenaemia during pregnancy, or within two months of the birth of their children, Schweitzer and his colleagues found a significant incidence of vertical transmission. Of twenty-three women who had hepatitis, twelve gave birth to infants who developed Australia antigenaemia, some of which began, not at delivery, but within weeks or months afterwards⁶. In each tested instance the babies had the same subtype antigen as their mothers⁷. The children did not have signs of hepatitis other than mild elevations of serum glutamic pyruvic transaminase (SGPT). Their development was normal although the antigenaemia has persisted for up to 23 months. Of fourteen mothers in this series who were chronic antigen carriers one gave birth to a baby who became a chronic carrier. The frequency of transmission from asymptomatic mothers varies in different locations. In contrast to Japan (7/12), Greece (2/11) and California (1/14), in Denmark⁸ none of the babies of eighty-one carrier mothers became positive, and in Pakistan one out of eighteen infants of asymptomatic carrier mothers developed the antigen⁹. These studies indicate that Au transmission from mothers with hepatitis is common and in some places maternal transmission from chronic carriers does occur.

The source of Au infection sometimes can be identified by immunological studies. The surface of the antigen is a composite of determinants which are immunologically distinct¹⁰⁻¹⁵. Persons infected with material containing Au of a certain immunological combination, or subtypes, will develop those subtypes¹⁶. The same subtype persists in both hepatitis patients and chronic carriers for as long as they have the antigen, with rare exceptions^{17,18}. We have used immunological subtyping to study relationships between Au carried by different members of the same family.

The sera used in this study were collected together with pedigrees in eighteen villages in south central Bougainville¹⁹. They represented 85% of the healthy residents over the age of 2 yr. It was assumed that asymptomatic people with antigenaemia were chronic carriers. There was an Au carrier

rate of 10.2% on Bougainville and family clustering had been observed in the initial testing of these sera. The distribution of Au had previously been subjected to segregation analysis which was compatible with autosomal recessive inheritance¹⁹. The observed family clustering could also be explained by intrafamily transmission of an infectious agent or maternal transmission as suggested by Turner *et al.*⁴. If this were true, then all members of a family should have the same subtype. The presence of multiple subtypes in the same family would rule against the family infection hypothesis. Sera for testing were selected from families with more than one antigen carrier so that a comparison between family members could be made.

The subtyping was done by immunodiffusion in a standard seven-hole pattern cut in 1.1% agarose in veronal buffer, pH 8.2 (ref. 20). In preliminary testing for d and w the antiserum was put in the centre well and a control Au which carried the determinant complementary to the antiserum was put in the top and bottom wells. If a smooth line of identity was formed between the control antigen and the test antigen the test antigen was scored as positive for the determinant in question. If a spur was formed it was considered negative for the determinant. Absorbed antiserum was used to test for determinant y. Serum from a chimpanzee immunised with Au of subtype aywx was absorbed with adwx + y- antigen, leaving anti-y in the antiserum. This was used with a y positive antigen control. Antigens giving a line of identity were considered positive; those giving no line, negative.

A few unusual sera carried neither d nor y. All such d- y- antigens were retested with different dilutions of antisera and an additional anti-ad and anti-ay sera. All plates were dried and stained with azocarmine for better visualisation of precipitin lines.

Table 1 Australia Antigen Subtypes Found in Eighteen Villages on Bougainville

Village	Total Au	Subtype D (d+y-)		Subtype Y (d-y+)		Subtype A (d-y-)		Subtype DY (d+y+)	
		w+	w-	w+	w-	w+	w-	w+	w-
1	18	0	3	10	4	1	0	0	0
2	4	1	1	2	0	0	0	0	0
3	3	0	2	0	1	0	0	0	0
4	5	0	1	3	1	0	0	0	0
5	5	0	4	0	1	0	0	0	0
6	14	0	9	5	0	0	0	0	0
7	14	4	8	0	2	0	0	0	0
8	22	6	10	0	5	0	1	0	0
9	4	3	0	1	0	0	0	0	0
10	19	1	15	2	0	0	1	0	0
11	2	0	1	1	0	0	0	0	0
12	11	0	1	10	0	0	0	0	0
13	2	0	0	1	1	0	0	0	0
14	5	0	2	0	3	0	0	0	0
15	5	0	0	1	3	0	0	1	0
16	1	0	0	0	0	0	0	1	0
17	20	1	11	0	8	0	0	0	0
18	20	1	7	6	6	0	0	0	0
	174	17	75	42	35	1	2	2	0

All possible combinations of determinants d, y and w except d+y+w- (Table 1) were found in this population. Of the 174 Au samples typed, two contained both d and y. This is a rare event and has not been reported before in normal people who have not received transfusions. These determinants seemed to be on the same particle (S. Mazzur and S. Burgert, personal communication).

Villages 2, 11, 13 and 16 did not contain families in which there were more than one member with Au. The data from families in the remaining villages which have more than one member with antigen are shown in Table 2. Of the thirty-two families tested there were eleven families in which two different combinations of antigens were segregating.

Asymptomatic carriers living in the same household can have different subtypes of Australia antigen (Table 2). This means

that these carriers have not been infected by each other nor from the same source. This rules out simple household infection or maternal transmission as an explanation for all the observed family clustering in these families.

A special case of family infection does, however, seem to occur. In families where the mother was an asymptomatic carrier, the twenty-two children who were carriers with a single exception had the same subtype as their mother. Among the twelve families where the mother was positive, one had mixed subtypes, while among the twenty families in which the mother did not have Au, ten families had antigen of different subtypes. These reflected the subtypes in the general

bution of Australia antigen in several populations. Segregation analysis of the distribution of Au in the same Bougainville sera which were used in this study revealed the segregation pattern expected of autosomal recessive inheritance. The vertical transmission reported in this communication is compatible with the genetic hypothesis since the genetic analysis was applied also to matings in which neither parent was a carrier of Au. There would be no maternal effect in these families. There is not an occult maternal effect due to undetected levels of antigen in the mothers' serum because the subtypes of the children do not, in general, match each other as they would in such a case.

Table 2 Distribution of Australia Antigen Subtypes in Families where More than One Member has the Antigen in the Blood

Village	Father	Mother	Sibs				
1	0	w+d-y+	w+d-y+	w+d-y+	w+d-y+		
3	0	w-d+y-	0	w-d+y-	0		
6	0	w-d+y-	w-d+y-				
7	0	w+d+y-	0	w+d+y-	w+d+y-		
9	0	w-d+y-	0	w-d+y-	w-d+y-	0	
10	NA	w-d+y-	0	0	w-d+y-		
12	NA	w+d-y+	w+d-y+	w+d-y+	w+d-y+	w+d-y+	0
12	NA	w+d-y+	w+d-y+	0			
14*	0	w-d+y+	w-d+y-	0	w-d-y+		
17	NA	w-d+y-	0	w-d+y-	w-d+y-		
18	NA	w-d+y+	0	w-d+y+	0	0	
18	NA	w+d-y+	w+d-y+	0	w+d-y+		
8	w+d+y-	NA	0	w+d+y-			
8*	w-d+y-	0	w-d-y+	0			
8*	w-d+y-	0	0	w+d+y-			
8	w-d+y-	NA	w-d+y-	0			
10*	w-d-y-	0	0	w-d+y-	0	0	
17*	w-d+y-	0	w-d-†	0			
4*	0	0	0	0	0	w-d-y+	w+d-y+
5*	0	0	w-d+y-	w-d-y+			
6	NA	0	0	w-d+y-	w-d+y-		
6*	0	0	w+d-y+	w-d+y-			
7*	NA	0	w+d+y-	w-d+y-	w-d+y-	w-d+y-	w-d+y-
8*	NA	0	w-d+y-	w+d+y-	0	w-d+y-	0
10	0	0	w-d+y-	w-d+y-	w-d+y-	0	w-d+y-
10*	0	0	0	w+d+y-	w-d+y-	0	
12	NA	NA	0	w+d-y+	w+d-y+		
12	0	NA	w+d-y+	w-d-y+			
15	0	0	w-d-y+	w-d-y+			
17	0	0	w-d-y+	w-d-y+	w-d-y+		
18	0	0	w-d+y-	0	w-d+y-		
18	0	0	w-d+y-	0	w-d+y-	0	

* Indicates families with mixed subtypes. NA indicates not available.

† Insufficient amount to test for y.

population of the village. Among the six families in which the father was positive, there were mixed subtypes in four of the families. Children with Au from families where either the mother but not the father, or the father but not the mother, carried the antigen were compared using Fisher's exact test (two-tailed). The difference in distribution was significant ($P=0.006$). When the families in which either parent was not available for testing were excluded from the analysis, the differences were still significant ($P=0.007$). The same test was used to compare the number of siblings of matching subtype in families with Au carrier and Au negative mothers. This difference was significant ($P=0.005$).

These data indicate that vertical transmission from asymptomatic mothers to their asymptomatic children occurs in this environment. Horizontal transmission with the production of carriers must also occur within this population since maternal transmission cannot account for all of the observed carriers.

Genetic predisposition to the chronic asymptomatic carrier state has been proposed by Blumberg^{19,21} and confirmed by Ceppellini¹ on the basis of segregation analysis of the distri-

Women who become Au carriers by virtue of genetic susceptibility can be expected to transmit the antigen to some of their children whether or not the children are homozygous for the postulated recessive Au susceptibility gene. Thus, a new maternal transmission line can be established from a genetically susceptible woman whose own mother was not a carrier provided the susceptible woman is exposed to infection. The interaction of the genetic predisposition to the carrier state and maternal transmission could be an important source of Au carriers. Further, the interaction of these two mechanisms, one of which is dependent on the survival and reproduction of women who are carriers, would provide a very rapid amplification of the number of carriers of Au in an environment where such a carrier state is advantageous. Such a selective advantage has been suggested by Blumberg *et al.*²²

Not all children of mothers who are Au carriers become carriers themselves. There are factors, either genetic or environmental or both, which influence the outcome of the maternal-child relationship. We suggest that other, perhaps undiscovered, human pathogens survive by the mechanisms

described here and are usually expressed as disease when passed horizontally or after long incubation. Techniques developed through the study of Australia antigen as well as epidemiological patterns disclosed by these studies could serve as models for the investigation of these agents. It has been suggested that other infectious agents may exist that have properties similar to Australia antigen. These have been termed Icrans²³.

Silent maternal transmission with the production of asymptomatic carriers seems to provide a reliable mechanism for virus survival particularly in small populations where the number of new susceptible individuals is limited. The carrier state maintains the infectious agent over long periods of time and the maternal-child interaction provides exposure of new susceptibles. In contrast, many acute viral infections spread quickly, exhaust the availability of susceptible hosts and disappear from isolated populations.

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- ¹ Ceppellini, R., Bedarida, G., Carbonara, A. O., Trinchieri, G., and Filippi, G., *Atti Convegno Farmitalia: Antigene Australia ed Epatite Virale*, 53 (Minerva Medica, Torino, 1970).
- ² Blumberg, B. S., *Bull. Acad. Med., Toronto*, **45**, 45 (1972).
- ³ Okochi, K., Mayumi, M., and Yishioka, K., in *Proc. third int. Symp. Princess Takamatsu Cancer Research Fund, Tokyo* (November 1972).
- ⁴ Turner, G. C., Field, A. M., Lasheen, R. M., Todd, R. McL., White, G. B. B., and Porter, A. A., *Archs. Dis. Child.*, **46**, 616 (1971).
- ⁵ Merrill, D. A., Dubois, R. S., and Kohler, P. F., *New Engl. J. Med.*, **287**, 1280 (1972).
- ⁶ Schweitzer, I. L., Wing, A., McPeak, C., and Spears, R. L., *J. Am. med. Ass.*, **220**, 1092 (1972).
- ⁷ Schweitzer, I. L., Edwards, V. M., Peters, R. L., and Mosley, J. W., *Gastroenterology*, **62**, 808 (1972).
- ⁸ Skinhøj, P., Olesen, H., Cohn, J., and Mikkelsen, M., *Acta Path. Microbiol. Scand.*, **B80**, 362 (1972).
- ⁹ Aziz, M., Kahn, G., Khanum, T., and Siddiqui, A., *J. infect. Dis.*, **127**, 110 (1973).
- ¹⁰ Levene, C., and Blumberg, B. S., *Nature*, **221**, 195 (1969).
- ¹¹ Raunio, V., London, W. T., Sutnick, A. I., Millman, I., and Blumberg, B. S., *Proc. Soc. exp. Biol. Med.*, **134**, 548 (1970).
- ¹² Kim, C. Y., and Tilles, J. G., *J. infect. Dis.*, **123**, 618 (1971).
- ¹³ Le Bouvier, G., *J. infect. Dis.*, **123**, 671 (1971).
- ¹⁴ van Kooten Kok-Doorschodt, H. J., van den Akker, R., and Gispert, R., *J. infect. Dis.*, **126**, 117 (1972).
- ¹⁵ Bancroft, W. H., Mundon, F. K., and Russell, P. K., *J. Immun.*, **109**, 842 (1972).
- ¹⁶ Le Bouvier, G. L., McCollum, R. W., Hierholzer, W. J., Irwin, G. R., Krugman, S., and Giles, J. P., *J. Am. med. Ass.*, **222**, 928 (1972).
- ¹⁷ Schmidt, N. J., Roberts, R. R., and Lennette, E. H., *Infect. Immunity*, **6**, 1 (1972).
- ¹⁸ Mazzur, S., and Blumberg, B. S., *Infect. Immunity*, **8**, 178 (1973).
- ¹⁹ Blumberg, B. S., Friedlaender, J. S., Woodside, A., Sutnick, A. I., and London, W. T., *Proc. natn. Acad. Sci. U.S.A.*, **62**, 1108 (1969).
- ²⁰ Mazzur, S., *Am. J. med. Tech.*, **38**, 343 (1972).
- ²¹ Blumberg, B. S., Melartin, L., Guinto, R. A., and Werner, B., *Am. J. hum. Genet.*, **18**, 594 (1966).
- ²² Blumberg, B. S., *Am. J. phys. Anthropol.*, **32**, 305 (1970).
- ²³ Blumberg, B. S., Millman, I., Sutnick, A. I., and London, W. T., *J. exp. Med.*, **134**, 320 (1971).

Lethal Effect of Near-ultraviolet Irradiation on Mammalian Cells in Culture

THE lethal and mutagenic effects of ultraviolet light of wavelength below 300 nm on bacterial and mammalian cells have been the subject of many research projects, and several laboratories are investigating the action of near-ultraviolet irradiation provided by black light (300 to 420 nm) on bacterial cells¹. There are, however, only a few studies on the effect of black light (near-ultraviolet) on mammalian cells^{2,3}. Several laboratories have reported effects of both visible fluorescent light, which does produce a significant amount of emission in the near-ultraviolet region, and black light on mammalian cells after the incorporation of bromodeoxyuridine (BUDR) into the cell nucleic acid³⁻⁷.

Experiments were initiated in this laboratory to study the effect of black light on mammalian cells in culture. Here we report that when mammalian cells in tissue culture medium were irradiated with black light, the cells were killed both reproductively (as evidenced by measuring clone-forming ability) and physiologically (as evidenced by staining with trypan blue dye).

The cells used were mouse 3T6 (ref. 8), Chinese hamster V79 clone 753-B-3M (ref. 9) and human D98/AH₂ (ref. 10). The cells were cultured in Dulbecco's modification of Eagle's medium¹¹ supplemented with 10% calf serum but without addition of antibiotics. The CO₂ tension for cell growth and light exposure was held at 10%. The light sources used were two General Electric F15T8 BLB integral filter black light tubes with emission in the 290 to 420 nm range peaking at 365 nm¹². Light exposures were carried out in covered Falcon 3002 60 mm dishes containing 3.5 ml medium with no phenol red or serum. The culture dish lids excluded irradiation below 300 nm¹³. Exposure intensity was 5 $\mu\text{W mm}^{-2}$ on the culture dish lid and 4 $\mu\text{W mm}^{-2}$ at the cell surface as measured by a Blak-ray meter J-221 (Ultraviolet Products, Inc.). Temperature was monitored and regulated to avoid any effect due to heating by the light source.

When mouse 3T6 fibroblast cells plated at 120 and 10⁴ cells per 60 mm dish were exposed to the BLB lights, 90% and 99% of the cells lost the ability to form clones during exposure times of 90 and 130 min respectively (Fig. 1a). The plating efficiency decreased less rapidly if the initial inoculum was 10⁵ cells. When 10⁶ cells were plated, no appreciable loss of plating efficiency was detected.

Similar density-dependent loss of plating efficiency was found with Chinese hamster V79 and human D98/AH₂ cells (Fig. 1b and c). V79 cells were inactivated at a slower rate than 3T6 cells when 120 and 10⁴ cells were plated per dish. No loss of plating efficiency was detected in dishes plated with 10⁵ or 10⁶ cells. D98/AH₂ cells were as sensitive to BLB as 3T6 except that the loss of plating efficiency was more rapid when 120 cells were plated per dish.

It seems that the final slopes of the inactivation curves, where loss of plating efficiency did occur, were similar for each cell line. The shoulders in the curves became more pronounced as cell density was increased.

The above experiments measured the loss of the cells' ability to form clones, that is, the amount of reproductive death. In order to ascertain whether black light killed the cells physiologically as well, we measured the ability of the cells to exclude the dye trypan blue which stains physiologically non-viable cells. Dishes containing 3,000 cells each were exposed to black light at 4 $\mu\text{W mm}^{-2}$ for 90 min and returned to a dark incubator. At various intervals after the exposure, trypan blue was added and the number of cells without dye was counted.

When 3T6 cells were used, approximately 90% of the cells lost their ability to exclude the dye during a 2 to 4 h period (Fig. 2a). For V79 cells the loss occurred between 6 to 8 h

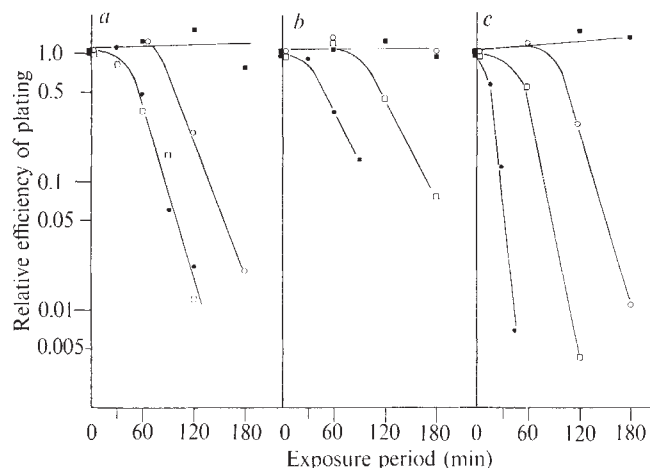


Fig. 1 The cells were trypsinised and plated in medium containing serum, but no phenol red, 18 h before exposure to $4 \mu\text{W mm}^{-2}$ black light. Twenty minutes prior to irradiation, the medium was removed and replaced with medium free of both phenol red and serum. The dishes were placed in the exposure chamber under 10% CO_2 for 20 min before the light was turned on. At various intervals dishes containing 120 cells were removed from the light and medium was replaced with fresh medium with serum. Dishes with 10^4 , 10^5 and 10^6 cells were subcultured and diluted into dishes containing fresh medium with serum. The subculture procedure itself does not alter the viability of the cells. All dishes were incubated at 37°C for 7 to 10 d in the dark. The dishes were then stained with haematoxylin and colonies counted. The result is expressed in relative efficiency of plating, or the number of colonies formed divided by number of cells inoculated initially, with the zero time value set equal to 1. ■, 10^6 ; ○, 10^5 ; □, 10^4 ; ●, 120 cells plated per 60 mm dish and exposed to black light. *a*, 3T6 cells; *b*, V79 cells; *c*, D98/AH₂ cells.

(Fig. 2*b*). We expected that at least 99% of the 3,000 D98/AH₂ cells would be killed after the 90-min exposure period (Fig. 1*c*) and the same proportion of cells (>99%) actually did lose the ability to exclude trypan blue within a 10-h period after exposure to black light (Fig. 2*c*).

Our results showed that black light killed human, mouse and Chinese hamster cells in Dulbecco's medium. Human and mouse cells were killed when up to 10^5 cells were plated per 60 mm tissue culture dish, and Chinese hamster cells were killed at densities up to 10^4 cells per dish. The killing effect was density dependent. When 10^6 cells were plated, no decrease in viability was observed.

At the molecular level, the effects of far-ultraviolet (< 300 nm) and near-ultraviolet (300 to 420 nm) on biological systems are quite different¹⁴. Far-ultraviolet leads to lethality by directly altering the DNA through dimer formation. Near-ultraviolet, at the dosage used in these experiments, does not produce dimers in bacterial systems¹. A possible chromophore may be present in the respiratory chain which absorbs energy in wavelengths above 300 nm¹⁴. Recombinationless bacterial mutants are highly sensitive to near-ultraviolet, suggesting that DNA may be an indirect target of the action of near-ultraviolet¹. One cannot rule out other possible chromophores which may act indirectly to produce the results reported here. It is also conceivable that toxic photoproducts are produced in the medium which when taken up by the cells leads to lethality. Our finding that the cell killing effect is density dependent could be due to the fact that when more cells were present, the amount of photoproducts absorbed by each cell was correspondingly less. This possibility is compatible with the shoulders observed in the inactivation curves (Fig. 1). No cell killing would be observed until the increase in toxic products was adequate to saturate each cell to a level sufficient to inactivate it. In a separate manuscript (unpublished) we shall show that after components of tissue culture medium are exposed to near-ultraviolet, toxic photoproducts are

formed which are primarily responsible for the type of mammalian cell inactivation as reported here.

The black light tubes used in this study emit in the 290 to 420 nm region. Although the peak of emission is relatively sharp¹², the responsible wavelengths for the killing observed here remain uncertain. Experiments are currently in progress with monochromatic light to determine the wavelengths which are most effective.

A better understanding of the effect of light irradiation in the 300 to 420 nm region on mammalian cells is needed. Visible fluorescent light, which illuminates virtually every laboratory, contains a significant amount of emission in this region. Puck and Kao⁴ devised a technique utilising BUdR and visible fluorescent light for the selection of auxotrophic Chinese hamster mutant cells after treatment with mutagens. Chu *et al.*³ reported that such selective techniques had highly mutagenic action. Visible fluorescent light increased the amount of polyoma virus induction nine-fold⁷. The near-ultraviolet region of visible fluorescent light can kill mammalian cells in culture (unpublished). The finding that near-ultraviolet produced by black light can effectively kill mammalian cells in tissue culture medium, as reported here, further emphasises the fact that considerable caution should be exercised in experiments involving the irradiation of mammalian cells with visible fluorescent light or black light. In addition, since black light is widely used outside the laboratory, the possible mutagenic and carcinogenic action of 300 to 420 nm radiation also needs to be carefully examined.

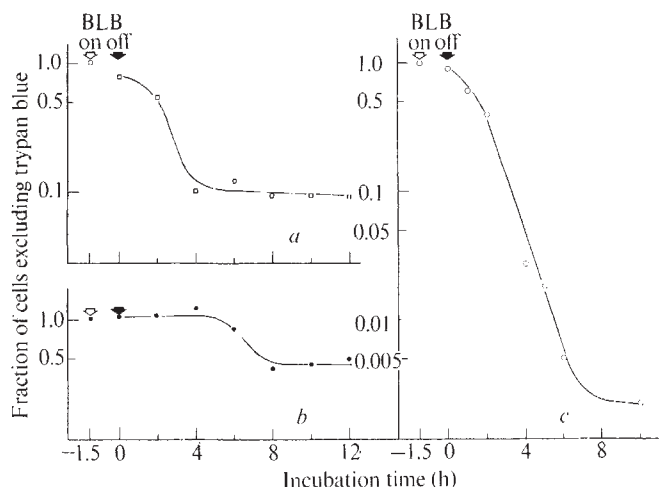


Fig. 2 3,000 cells were plated per 60 mm dish and exposed to $4 \mu\text{W mm}^{-2}$ black light as in Fig. 1. The cells were exposed to light for 90 min before being returned to incubation in the dark. At various intervals, trypan blue (0.5%) was added to the dishes for 5–7 min and the number of cells containing no dye was counted under $\times 40$ magnification. Symbols as in Fig. 1; *a*, 3T6 cells; *b*, V79 cells; *c*, D98/AH₂ cells.

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- ¹ Eisenstark, A., *Adv. Genet.*, **16**, 167 (1971).
- ² Klein, R. M., *Photochem. Photobiol.*, **6**, 841 (1967).
- ³ Chu, E. H. Y., Sun, N. C., and Chang, C., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3459 (1972).
- ⁴ Puck, T. T., and Kao, F. T., *Proc. natn. Acad. Sci. U.S.A.*, **58**, 1227 (1967).
- ⁵ Yang, S. J., and Hahn, G. M., *Photochem. Photobiol.*, **11**, 131 (1970).
- ⁶ Ben-Hur, E., and Elkind, M. M., *Mutation Res.*, **14**, 237 (1972).
- ⁷ Fogel, Marian, *Nature new Biol.*, **241**, 182 (1973).
- ⁸ Todaro, G. J., and Green, H., *J. Cell Biol.*, **17**, 299 (1963).
- ⁹ Shipley, W. V., Elkind, M. M., and Prather, W. B., *Radiat. Res.*, **47**, 437 (1971).
- ¹⁰ Szybalski, W., Szybalska, E. H., and Ragni, G., *Nat. Cancer Inst. Monogr.*, **7**, 75 (1962).
- ¹¹ Dulbecco, R., and Freeman, G., *Virology*, **8**, 396 (1959).
- ¹² *Black Light* (General Electric Publication TP-125, 1969).
- ¹³ Webb, R. B., and Lorenz, J., *J. Bact.*, **112**, 649 (1972).
- ¹⁴ Ferron, W. L., Eisenstark, A., and Mackay, D., *Biochim. biophys. Acta*, **277**, 651 (1972).

Control of Cell Surface Topography

ACCORDING to the fluid mosaic model of membrane structure¹, surface proteins are free to diffuse in a lipid matrix and thus to assume a random or homogenous distribution over the cell surface. In support of this model it has been shown that the distribution of glycoprotein receptors for the plant lectin concanavalin A (con A) is random in all cell types studied. In a variety of living systems it has also become clear that surface elements can be induced by exogenous agents to assume a non-random or heterogeneous distribution; for example, in virus transformed cells con A induces a clustering of con A binding sites. Recently, we and others have shown that cellular components sensitive to colchicine alkaloids (microtubules and perhaps other structures with similar pharmacological specificities) can affect the topography of certain surface elements. We shall review here the experimental evidence for systems in which topographical heterogeneity of specific surface elements (lectin binding sites and membrane transport carriers) can be induced and can be altered by colchicine. We shall then advance a general hypothesis involving specific interactions between surface proteins and intracellular colchicine binding proteins (CBP), which rationalises some apparent contradictions in this evidence and which indicates a molecular process at the level of the plasma membrane by which cells may respond to extracellular substances.

(1) When virus transformed fibroblasts are fixed with paraformaldehyde, the distribution of con A binding sites is random, as it is in non-transformed cells and as predicted by the fluid mosaic model. When con A is adsorbed to cells before fixation the distribution of con A binding sites in non-transformed cells is, however, still random, whereas in transformed cells the binding sites are gathered into clusters². These clusters are withdrawn from the edges of the cell but otherwise are themselves disperse (T. E. U., R. D. B., J. Z. Borysenko, and M. J. Karnovsky, unpublished). Incubation of transformed cells with 1 μ M colchicine before con A does not affect the formation of clusters, but the clusters are now loosely grouped into a large circumnuclear surface aggregate. These data suggest an enhanced mobility of con A binding sites after transformation which is further increased by alkaloid treatment.

(2) Treatment of lymphocytes with low concentrations of con A at 37° C leads to movement of con A binding sites into large aggregates known as "caps". At high concentrations of con A, however, no caps are formed during incubation at 37° C^{3,4}. Thus, con A binding sites seem to be immobilised after addition of the high concentrations of lectin. In parallel experiments where lymphocytes are cooled to 4° C (presumably dissolving microtubules) or treated with colchicine, con A leads to capping even at high con-

centrations⁵, indicating an increased mobility of con A binding site clusters in these cells.

(3) During phagocytosis particles are enveloped by cytoplasmic membrane which is then withdrawn into membrane-bound intracellular vesicles. Under suitable conditions more than 50% of the surface of polymorphonuclear leukocytes or alveolar macrophages can be internalised in this fashion. Using initial rates of purine and amino acid transport to assay the numbers of specific carrier proteins in the membrane, it has been shown that the same number of transport carriers are present before and after removal of a high proportion of the plasma membrane by phagocytic ingestion of latex beads⁶. Other experiments⁶ rule out the insertion of new carriers into the surface membrane. This preservation of transport sites indicates their separation into a topographically distinct area which does not undergo internalisation. When leukocytes are pretreated with colchicine, however, phagocytosis leads to a decrease in transport rate which parallels the degree of membrane internalisation⁷. Thus, it seems that the forces which exclude transport carriers from phagocytic areas do not operate after colchicine treatment.

(4) Morphological studies of non-phagocytosing leukocytes show that con A binding sites are distributed randomly on the cell surface (J. M. O., R. D. B., J. Z. Borysenko, and M. J. Karnovsky, unpublished). When con A and *Ricinus communis* agglutinin (RCA) receptors are titrated after the phagocytic ingestion of droplets of a paraffin oil emulsion it can, however, be shown that the specific activity of surface binding sites (defined as μ g lectin bound per 100 μ g membrane protein) is reduced by 40–60% (the reverse of case (3) above for transport proteins). Thus, during phagocytosis lectin binding sites are removed from the surface, apparently by concentration in the regions undergoing internalisation. After colchicine treatment, the removal of specific populations of lectin binding from the surface is greatly reduced, indicating an uncoupling of the process which links the concentrative movement of con A binding sites with the internalisation of phagocytic particles⁸.

The effects of colchicine on (1) and (2) as compared with (3) and (4) on movement of specific surface elements are in apparent contradiction. Thus, movement of con A binding site clusters induced by con A, as in cap formation, occurs only after colchicine treatment whereas the concentrative movements of transport carriers and lectin binding sites induced by phagocytosis do not occur after colchicine. This paradox can be resolved simply by the following hypothesis: for cross-linking by con A (1) and (2), clusters of con A binding sites must have sufficient mobility to be approximated by the lectin. This requires release from cellular restraints which tend to separate and/or direct them. Colchicine abolishes these restraints. On the other hand, the directed movements which occur during phagocytosis ((3) and (4)) require the attachment of receptors (or transport proteins) to these restraining cellular structures which themselves direct specific movement. Colchicine abolishes these attachments and thus the cell-directed movement of con A binding sites into (and probably certain transport proteins out of) membrane internalised by phagocytosis. Thus, in all cases colchicine abolishes the attachment of membrane proteins to mobile cellular structures, permitting their association by exogenous cross-linking agents on the one hand, but abolishing cell-directed movements on the other.

What is the nature of these colchicine-sensitive linking structures? In the cases so far examined their pharmacological specificity parallels that of microtubular proteins: sensitivity to colchicine, colcemid, and the chemically unrelated vinblastine, and insensitivity to the photochemical derivative, lumicolchicine. Operationally, therefore, the 'linkage element' behaves like a microtubular protein, but it is not yet proven that colchicine treatment alters membrane properties through its interaction with structures identified morphologically as microtubules. It is possible that contrac-

tile structures, perhaps microfilaments, may interact with these colchicine-sensitive elements in order to facilitate movements of the surface proteins.

Our hypothesis represents a synthesis of the fluid mosaic theory, which suggests a random distribution of surface proteins, with data from several experimental systems in which topographical heterogeneity can be produced. It is proposed that con A-receptor complexes can be aggregated into large caps if the receptors are not restrained in their movement patterns by colchicine-sensitive elements; and in addition that movement of con A binding sites can be directed into local regions by exogenous agents such as phagocytic particles if these linkage elements are intact, that is, cell-directed movements of con A binding sites and cross-linking by bound con A are in this sense mutually exclusive. Experiments which test this theory may allow more precise definition of the mechanisms by which changes in cell surface topography can be directed by intrinsic cellular structures in response to extrinsic forces.

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- ¹ Singer, S. J., and Nicolson, G. L., *Science, N.Y.*, **175**, 720 (1972).
- ² Rosenblith, J. Z., Ukena, T. E., Yin, H. H., Berlin, R. D., and Karnovsky, M. J., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1625 (1973).
- ³ Yahara, I., and Edelman, G. M., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 608 (1972).
- ⁴ Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., and Edelman, G. M., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1012 (1973).
- ⁵ Edelman, G. M., Yahara, I., and Wang, J. L., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1442 (1973).
- ⁶ Tsan, M. F., and Berlin, R. D., *J. exp. Med.*, **134**, 1016 (1971).
- ⁷ Ukena, T. E., and Berlin, R. D., *J. exp. Med.*, **136**, 1 (1972).
- ⁸ Oliver, J. M., Ukena, T. E., and Berlin, R. D., *Proc. natn. Acad. Sci. U.S.A.* (in the press).

Immune Complexes induce Selective Release of Lysosomal Hydrolases from Macrophages

IMMUNE complexes contribute to tissue damage in a variety of experimental and human diseases. Immune complexes induce arthritis¹ and nephritis^{2,3} in laboratory animals while lupus glomerulonephritis⁴ and possibly rheumatoid arthritis⁵ are examples of their effects in man. The mechanisms by which damage is initiated and perpetuated are poorly understood. In the acute stage of inflammation resulting from immune complexes many granulocytes are present. These cells have abundant lysosomes rich in acid hydrolases which when released can degrade various tissue components. Enzyme release may result from cell death or exocytosis of granules from viable cells. Henson⁶ and others^{7,8} have shown that granulocytes exposed to immune complexes *in vitro* release lysosomal enzymes while remaining viable, and presumably the same happens in Arthus reactions *in vivo*.

After the initial phase of immune complex-induced tissue injury, neutrophils are no longer prominent and other cells may play a role in the continuation of the inflammation.

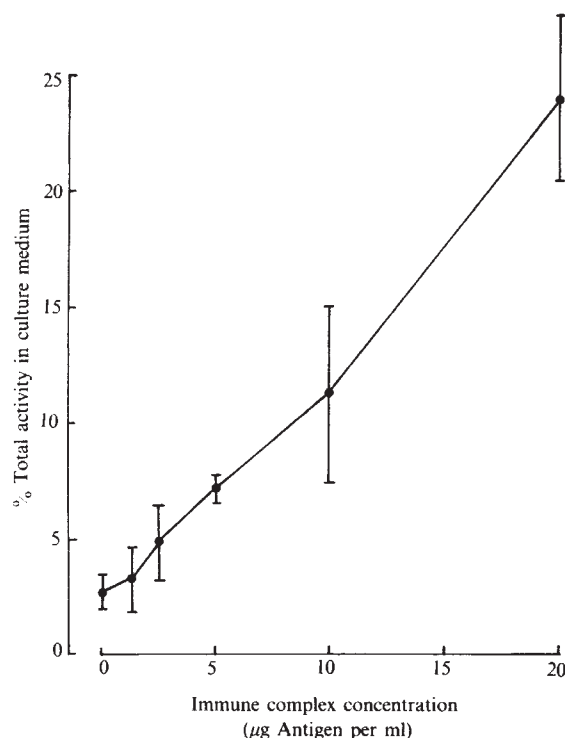


Fig. 1 The release of β -glucuronidase from macrophages exposed to increasing concentrations of immune complexes. Macrophages were collected by peritoneal lavage of outbred Swiss mice (LACA strain, SACL, London Road, Braintree, Essex) with 5 ml M199 (Grand Island Biological Co., Grand Island, New York) + 10% swine serum (Biocult Laboratories, Glasgow). 2 to 2.5×10^6 cells were plated in 48 mm plastic Petri dishes (Nunc, Jobling Laboratories Division, Stone, Staffordshire). After 30 min the cell sheets were washed four times with phosphate-buffered saline and then cultured in M199 + 10% swine serum at 37°C in a 5% CO_2 in air atmosphere for 48 h. Immune complexes were formed by interacting ferritin (Miles Laboratories) and rabbit anti-ferritin serum (Miles Laboratories). The point of equivalence was determined by quantitative precipitation¹⁶. The cells were exposed to increasing amounts of immune complexes for 6 h. At this time the culture medium was removed, centrifuged at 1,000 r.p.m. for 10 min in a No. 59548 swing-out head of a Mistral 6L centrifuge to pellet detached cells. The cell sheet was washed once with 0.9% NaCl (w/v) and then removed in 2.5 ml 0.9% NaCl (w/v) containing 0.1% Triton X-100 (w/v). All fractions were assayed for β -glucuronidase activity by the method of Talalay *et al.*¹⁷. Each value represents the mean $\pm$ s.d. of four separate plates.

Phagocytic cells such as blood-borne monocytes, tissue macrophages, mesangial cells of the kidney and A cells of synovium are present at sites of tissue damage in chronic inflammation associated with immune complexes. Moreover, mononuclear phagocytic cells are known to interact with immune complexes. Human monocytes have receptors for the Fc portions of IgG1 and IgG3⁹. Mouse peritoneal macrophages and rabbit alveolar macrophages take up immune complexes^{10,11}, as do renal mesangial cells¹² and A cells of rheumatoid synovium¹. The interaction of these phagocytic cells with immune complexes may also result in tissue damage. These cells contain lysosomal enzymes which can not only digest immune complexes but degrade connective tissue constituents when released from the cells¹³. Lysosomal hydrolases may again be released from macrophages by cell death¹⁴ or selectively discharged from viable cells (ref. 15 and P. D., R. Page, and A. C. A., unpublished). It has already been reported that macrophages take up and digest immune complexes *in vitro*¹⁰. We now present evidence that this process is accompanied by a selective release of macrophage hydrolases from viable cells into the culture medium.

Figure 1 shows the effect of increasing concentrations of an immune complex on the release of lysosomal enzymes from macrophages. After 6 h incubation in medium containing $5 \mu\text{g ml}^{-1}$ of immune complex, the concentration of β -glucuronidase in the medium is significantly greater than controls ($P < 0.01$). There is a dose-dependent release of

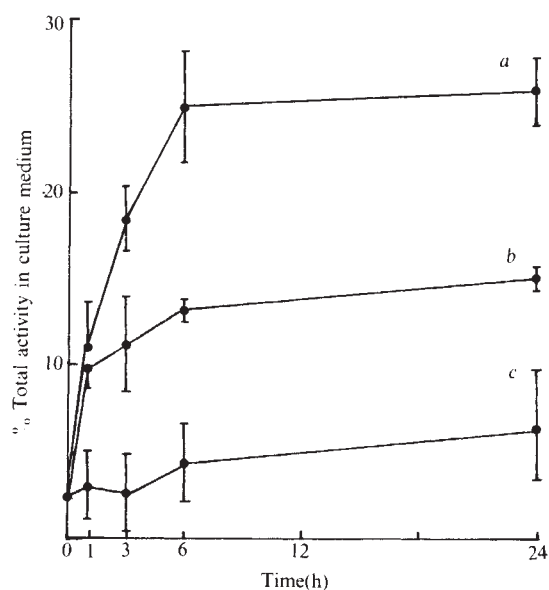


Fig. 2 The time dependence of macrophage β -glucuronidase release induced by immune complexes. The experiment was carried out in similar conditions to those described in Fig. 1 except that immune complexes were formed at equivalence from bovine serum albumin, Cohn Fraction V (Sigma Chemical Co., London) and rabbit anti-bovine serum albumin (BSA-anti-BSA). The latter was prepared by intramuscular inoculation of New Zealand White rabbits with 15 mg of antigen followed 3 weeks later by injection of 5 mg antigen intravenously. The animals were bled 10 d later. Each value represents the mean $\pm$ s.d. of four separate plates. a, $20 \mu\text{g ml}^{-1}$; b, $10 \mu\text{g ml}^{-1}$; c, control.

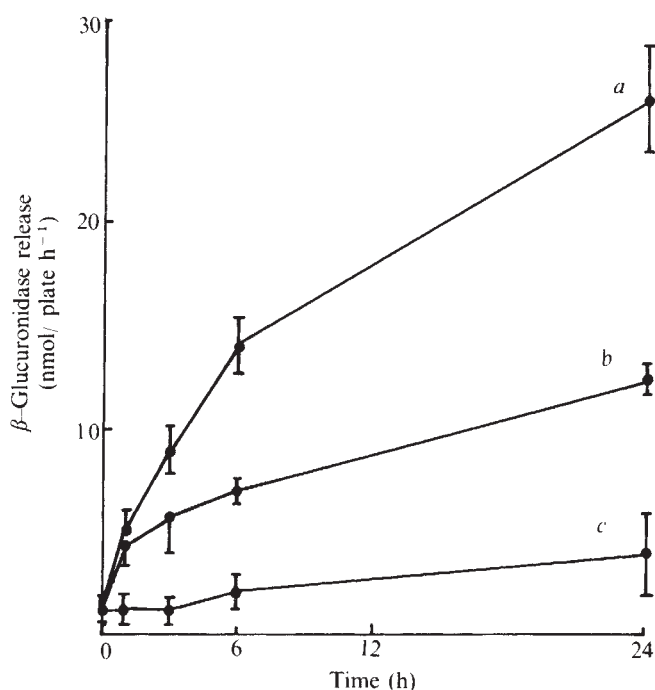


Fig. 3 The release of increasing amounts of β -glucuronidase from macrophages exposed to immune complexes for 24 h. Experimental conditions were identical to those described in Fig. 2. Each point represents the mean $\pm$ s.d. of four separate plates. a, $20 \mu\text{g ml}^{-1}$; b, $10 \mu\text{g ml}^{-1}$; c, control.

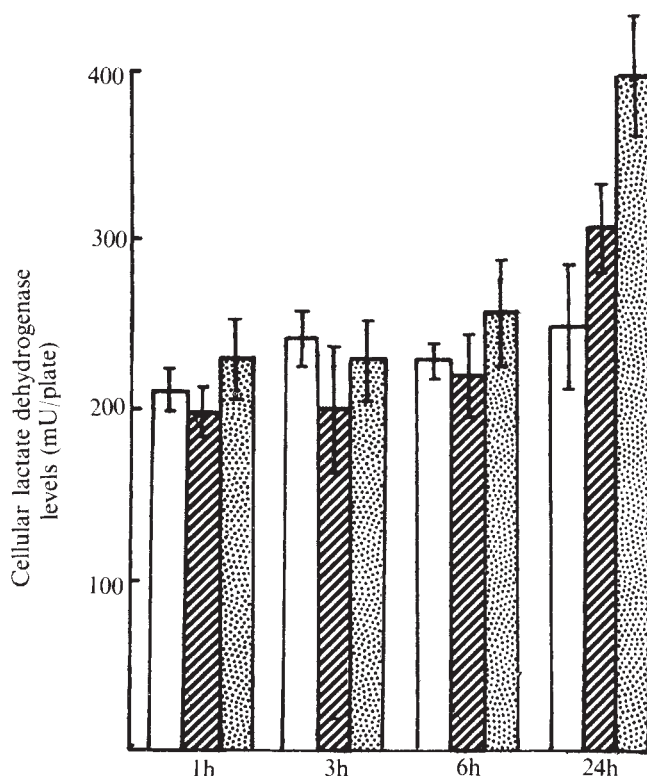


Fig. 4 The levels of lactate dehydrogenase (LDH) in macrophages exposed to immune complexes. Experimental conditions were identical to those described in Fig. 2. LDH was assayed using Biochemical Test Combination LDH-UV (Boehringer Corp. (London) Ltd). $\square$, Control; ▨ , immune complexes ($20 \mu\text{g ml}^{-1}$); ▤ , immune complexes ($10 \mu\text{g ml}^{-1}$).

enzyme rising to $24.0 \pm 3.5\%$ with $20 \mu\text{g ml}^{-1}$ of immune complex.

Release of the lysosomal enzyme is time dependent. In Fig. 2, the release of β -glucuronidase into the culture medium over 24 h with $10 \mu\text{g ml}^{-1}$ and $20 \mu\text{g ml}^{-1}$ of immune complex is shown. Release is demonstrable 1 h after contact and continues over the subsequent 23 h. Although the percentage of total β -glucuronidase activity in the culture medium plateaus after 6 h, the absolute amount continues to increase because of enzyme synthesis by the cells (Fig. 3). This indicates that depletion of intracellular acid hydrolase may act as a signal for increased enzyme synthesis.

Figure 4 shows that at no time during the 24 h period is there a significant decrease in cellular lactate dehydrogenase levels, compared with controls, of cultures exposed to immune complex ($P > 0.01$) in all instances. Moreover, cultures exposed to $10 \mu\text{g ml}^{-1}$ of immune complex for 24 h show a statistically significant ($P < 0.01$) increase in cellular LDH level compared with its control.

For the sake of brevity we have only presented data for β -glucuronidase, but a similar pattern of selective release has been demonstrated for other lysosomal hydrolases such as N-acetyl- β -D-glucosaminidase and β -galactosidase; this information will be published in detail elsewhere.

It is now clear that both granulocytes and macrophages, phagocytic cells associated with acute and chronic inflammation respectively, can selectively release lysosomal hydrolases with no detectable loss of viability. This phenomenon may provide an explanation for the ability of macrophages to cause tissue damage and degradation at sites of chronic inflammation while retaining their viability over prolonged periods of time. This interpretation is supported by evidence that other agents inducing chronic inflammation, such as Group A streptococcal cell walls, also cause selective release of lysosomal hydrolases from viable macrophages *in vitro* (P. D., R. Page, and A. C. A., unpublished).

As immune complexes and macrophages are both present in the joints of patients with rheumatoid arthritis, release of macrophage hydrolases could play an important part in degradation of cartilage and other connective tissue changes observed in this disease. The role of immune complexes and macrophages in other chronic inflammatory conditions also deserves further study.

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- ¹ Hollister, J. R., Liang, G. C., and Mannik, M., *Arthritis Rheum.*, **16**, 10 (1973).
- ² Pincus, T., Haberkern, R., and Christian, C. L., *J. exp. Med.*, **127**, 819 (1968).
- ³ Unanue, E. R., and Dixon, F. J., *Adv. Immun.*, **6**, 1 (1967).
- ⁴ Koffler, D., Schur, P. H., and Kunkel, H. G., *J. exp. Med.*, **126**, 607 (1967).
- ⁵ Bonomo, L., Tursi, A., Trizio, D., Gillardi, U., and Dammacco, F., *Immunology*, **18**, 557 (1970).
- ⁶ Henson, P. M., *J. Immun.*, **107**, 1535 (1971).
- ⁷ Hawkins, D., *J. Immun.*, **108**, 310 (1972).
- ⁸ Weissmann, G., Zurier, R. B., Spieler, P. J., and Goldstein, I. M., *J. exp. Med.*, **134**, 149s (1971).
- ⁹ Hay, F. C., Torrigiani, G., and Roitt, I. M., *Eur. J. Immun.*, **2**, 257 (1972).
- ¹⁰ Steinman, R. M., and Cohn, Z. A., *J. Cell Biol.*, **55**, 616 (1972).
- ¹¹ Phillips-Quagliata, J. M., Levine, B. B., Quagliata, F., and Uhr, J. W., *J. exp. Med.*, **133**, 589 (1971).
- ¹² Okuda, R., Kaplan, M. H., Cuppage, F. E., and Heymann, W., *J. Lab. clin. Med.*, **66**, 204 (1965).
- ¹³ Dingle, J. T., Poole, A. R., Lazarus, G. S., and Barrett, A. J., *J. exp. Med.*, **137**, 1124 (1973).
- ¹⁴ Allison, A. C., Harrington, J. S., and Birbeck, M., *J. exp. Med.*, **124**, 141 (1968).
- ¹⁵ Weissmann, G., Dukor, P., and Sessa, G., *Immunopathology of Inflammation* (edit. by Forscher, B., and Houck, J. C.), 107 (Excerpta Medica, Amsterdam, 1971).
- ¹⁶ Kabat, E. A., *Experimental Immunochemistry* (edit. by Kabat, E. A., and Mayer, M. M.) (Charles C. Thomas, Springfield, Illinois, 1961).
- ¹⁷ Talalay, P., Fishman, W. H., and Huggins, C., *J. biol. Chem.*, **166**, 757 (1946).

Vitamin A Deficiency enhances Binding of Benzo(a)pyrene to Tracheal Epithelial DNA

THE relationship between carcinogen action and the state of cellular differentiation in target tissues is still unclear. Vitamin A controls the state of differentiation in many epithelial tissues¹, including those of the respiratory tract^{2,3}. Vitamin A can also modify the process of carcinogenesis in many tissues; in a variety of experiments, the results obtained have often been influenced by experimental variables such as the animal species, the time and route of vitamin A administration, and the morphological type and the tissue site of tumour induced⁴⁻¹⁰. In the respiratory epithelium⁹ vitamin A deficiency causes basal cell hyperplasia and squamous metaplasia. In order to study the possible relationship of the altered state of differentiation of respiratory epithelium to chemical carcinogenesis in this tissue, we have studied the binding of tritiated benzo(a)pyrene (³H-BP) to DNA in tracheal epithelial cells of normal and vitamin A deficient hamsters. The binding of carcinogens to DNA has been indicated as a potentially crucial step in the carcinogenic process¹¹⁻¹³. For polynuclear hydro-

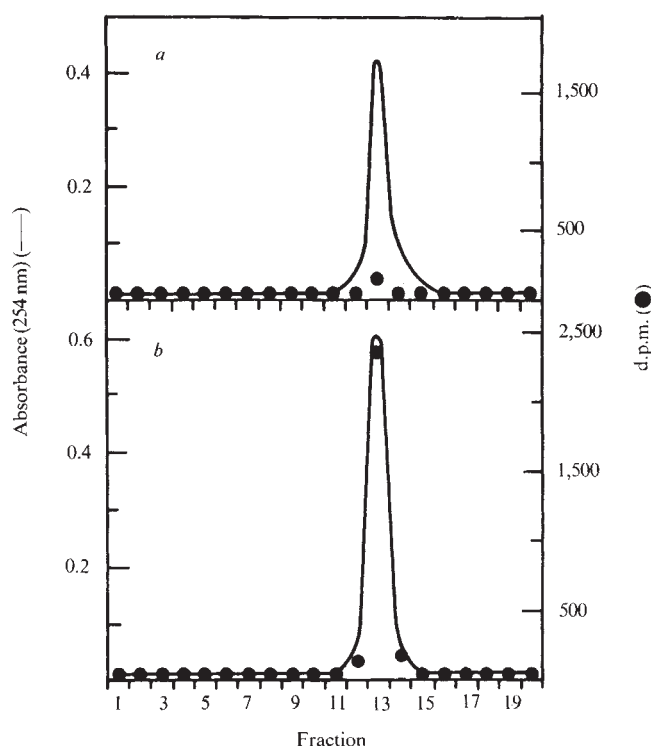


Fig. 1 CsCl gradient profiles of DNA purified from tracheal epithelium isolated from, *a*, normal and, *b*, vitamin A deficient hamsters. Isolated tracheas were maintained *in vitro* in L-15 medium containing 8.0×10^{-7} M ³H-BP (20 μ Ci ml⁻¹, specific activity 25 Ci mmol⁻¹, Amersham-Searle). Following 3 h incubation, the tracheal epithelial cells were scraped from supporting structures and a crude preparation of DNA was obtained by phenol extraction and precipitation with 50% ethanol. The precipitate was extracted with ether, benzene, and chloroform-methanol (3:1), and then sequentially digested with RNase (Worthington Biochemicals) and Pronase (Calbiochem). The aqueous solution of DNA was extensively dialysed and then banded on CsCl gradients (Beckman SW56 rotor, 35,000 r.p.m., 66 h, 25° C). Gradients were fractionated into 0.2 ml portions, and absorbance and radioactivity were determined on each fraction. The absorbance of the gradient effluent was continuously monitored at 254 nm, as shown by the continuous line; radioactivity (d.p.m.) is indicated as points.

carbons such as benzo(a)pyrene, this binding is presumed to be the ultimate result of metabolism to reactive carcinogenic forms¹⁴⁻¹⁶; the extent of binding may be a measure of the carcinogenic potential towards a given tissue.

We report here the relative binding of ³H-BP to tracheal epithelial DNA of vitamin A deficient and pair-fed normal hamsters under conditions in which isolated tracheas were incubated *in vitro* in the presence of ³H-BP. Hamsters were made vitamin A deficient by maintenance on a vitamin A deficient diet starting at day 10-14 after birth; controls were pair-fed with the same diet supplemented with vitamin A (100 μ g *all-trans* retinyl acetate, twice a week, by stomach tube)⁹. Tracheas were removed from the vitamin A deficient and pair-fed control hamsters and the isolated tracheas were incubated *in vitro* in L-15 medium containing ³H-BP under conditions which preserved biochemical function and morphology¹⁷. Following incubation, the tracheas were washed extensively with phosphate buffered saline and the tracheal epithelial cells were scraped from supporting structures. DNA was isolated from the epithelial cells, and the purified DNA was banded in CsCl gradients as previously reported¹⁸. The results are shown in Fig. 1 and Table 1. Substantially greater quantities of radioactivity were associated with DNA prepared from vitamin A deficient tracheas. When isolated tracheas were incubated *in vitro* in the presence of ³H-BP and equimolar amounts of 7,8-benzoflavone (7,8-BF), an inhibitor of the aryl hydro-

carbon hydroxylase system^{19,20}, the binding of ³H-BP to DNA was reduced (Table 1). This result strongly suggests that the extent of benzo(a)pyrene binding to DNA depends largely on the ability of tracheal epithelial cells to metabolise it. The extent to which the increased binding of benzo(a)pyrene to tracheal DNA in vitamin A deficient animals depends on altered activity of microsomal aryl hydrocarbon hydroxylase enzymes or on other altered cellular conditions, such as altered cellular membrane permeability or increased DNA synthesis, has yet to be established.

Table 1 Specific Activity of ³H-BP bound to DNA of Tracheal Epithelial Cells during *in vitro* Incubation

Vitamin A status of animals	Addition to incubation medium		³ H-BP and 7,8-BF Exp. 2
	³ H-BP Exp. 1	Exp. 2	
Normal	6.0	13.4	1.3
Deficient	25.0	76.2	1.3

The data are expressed as d.p.m. per μ g DNA. The μ g of DNA and d.p.m. was determined from the peak DNA fraction of CsCl gradients. For each value presented in the table, DNA was prepared from two tracheas and pooled before purification on CsCl gradients. The animals were made vitamin A deficient by maintenance on a vitamin A deficient diet, starting on day 14 after birth in Exp. 1 and on day 10 after birth in Exp. 2.

The cellular location of the increased binding of benzo(a)pyrene in vitamin A deficient tracheal epithelium has recently been demonstrated by quantitative light microscope autoradiographic studies. C. C. H. *et al.* (unpublished observations), using quantitative techniques²¹, have demonstrated that in vitamin A deficient tracheas there is a higher binding of ³H-BP to macromolecular structures of basal cells located in squamous metaplastic areas than to basal cells located in normal areas. These studies will be reported elsewhere in full detail.

The aim of the above experiments was to study the effects of a potentially co-carcinogenic condition, namely vitamin A deficiency, on the extent of binding of benzo(a)pyrene to hamster tracheal DNA. It has not yet been shown in parallel long term animal studies, however, that vitamin A deficiency determines a state of high carcinogenic susceptibility in this tissue and in this species. In an analogous *in vivo* experiment conducted on rats which had been treated with the carcinogenic hydrocarbon 3-methylcholanthrene, preliminary results suggest that there is a higher incidence or an earlier appearance of respiratory tract tumours in animals fed a diet with a subnormal vitamin A content, compared with animals maintained on a normal vitamin A diet (P. Nettesheim, personal communication). Similar results have also been reported for colonic tumour induction in rats maintained on diets with subnormal vitamin A content^{22,23}. The above results suggest that determination of the extent of binding of benzo(a)pyrene to DNA during short term organ culture of isolated tracheas may be applicable as a method for screening potential states (or potential individuals) with increased susceptibility to respiratory carcinogenesis.

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- ¹ Wolbach, S. B., and Howe, P. R., *J. exp. Med.*, **47**, 753 (1925).
- ² Harris, C. C., Sporn, M. B., Kaufman, D. G., Smith, J. M., Jackson, F. E., and Saffiotti, U., *J. natn. Cancer Inst.*, **48**, 743 (1972).
- ³ Wong, Y., and Buck, R., *Lab. Invest.*, **24**, 55 (1971).
- ⁴ Saffiotti, U., Montesano, R., Sellakumar, A. R., and Borg, S. A., *Cancer*, **20**, 857 (1967).
- ⁵ Chu, E. W., and Malmgren, R. A., *Cancer Res.*, **25**, 884 (1965).
- ⁶ McMichael, H., *Cancer Res.*, **25**, 947 (1965).
- ⁷ Davies, R. E., *Cancer Res.*, **27**, 237 (1967).
- ⁸ Polliack, A., and Levij, I. S., *Cancer Res.*, **29**, 327 (1969).
- ⁹ Polliack, A., and Ben-Sasson, Z., *J. natn. Cancer Inst.*, **48**, 407 (1972).
- ¹⁰ Bollag, W., *Cancer Chem. Rep.*, **55**, 53 (1971).
- ¹¹ Brookes, P., and Lawley, P. D., *Nature*, **202**, 781 (1964).
- ¹² Sporn, M. B., and Dingman, C. W., *Nature*, **210**, 531 (1966).
- ¹³ Brookes, P., *Cancer Res.*, **26**, 1994 (1966).
- ¹⁴ Gelboin, H. V., *Cancer Res.*, **29**, 1272 (1969).
- ¹⁵ Duncan, M. E., and Brookes, P., *Internat. J. Cancer*, **6**, 496 (1970).
- ¹⁶ Wang, I. Y., Rasmussen, R. E., and Crocker, T. T., *Biochem. biophys. Res. Commun.*, **49**, 1142 (1972).
- ¹⁷ Kaufman, D. G., Baker, M. S., Harris, C. C., Smith, J. M., Boren, H., Sporn, M. B., and Saffiotti, U., *J. natn. Cancer Inst.*, **49**, 783 (1972).
- ¹⁸ Kaufman, D. G., Genta, V. M., Harris, C. C., Smith, J. M., Sporn, M. B., and Saffiotti, U., *Cancer Res.*, **33**, 2837 (1973).
- ¹⁹ Diamond, L., and Gelboin, H. V., *Science, N.Y.*, **166**, 1023 (1969).
- ²⁰ Wiebel, F. J., Leutz, J. C., Diamond, L., and Gelboin, H. V., *Archs. Biochem. Biophys.*, **144**, 78 (1971).
- ²¹ Harris, C. C., Kaufman, D. G., Sporn, M. B., Boren, H., Jackson, F., Smith, J. M., Pauley, J., Dedick, P., and Saffiotti, U., *Cancer Res.*, **33**, 2842 (1973).
- ²² Rogers, A. E., Herndon, B. J., and Newberne, P. M., *Cancer Res.*, **33**, 1003 (1973).
- ²³ Newberne, P. M., and Rogers, A. E., *J. natn. Cancer Inst.*, **50**, 439 (1973).

Evidence for a Junctional Effect of Lead on Neuromuscular Function

THE peripheral neuromuscular pathology of lead has been extensively described in the clinical literature, but the mechanism of lead toxicity which results in neuromuscular impairment is not known¹. Early work attempted to locate the site of a toxic lesion in the muscle and showed changes in muscle inorganic phosphate and creatine phosphate levels after lead treatment^{2,3}. Experiments on the superior cervical ganglion, however, suggested that lead affected synaptic transmission by lowering the amount of acetylcholine released on preganglionic stimulation⁴. In order to separate the effects of lead on nerve from effects on muscle we have carried out experiments using the isolated phrenic nerve-diaphragm preparation.

Male Sprague-Dawley rats, weighing 120 to 135 g, were anaesthetised and the diaphragms with both phrenic nerves attached were removed and separated into two hemidiaphragms, each with a nerve trunk attached. The nerve-muscle preparation was similar to that originally described by Bulbring⁵, modified so that preparations could be stimulated separately through either the nerve or muscle. One hemidiaphragm with its phrenic nerve was exposed to lead, as lead chloride, in a range of concentrations from 1×10^{-5} M to 1×10^{-4} M. Its pair was run simultaneously as a control. (Krebs-Ringer bicarbonate solution was used in all experiments, with the following composition: CaCl₂, 2.5 mM; KCl, 3.5 mM; NaCl, 117 mM; MgSO₄, 1.2 mM; KH₂PO₄, 1.2 mM; NaHCO₃, 56 mM; glucose, 11 mM.)

Maximum force of contraction of an isometric twitch for nerve and muscle stimulation was measured by a force transducer throughout the experiment. (Maximum force of contraction was found to be developed when the hemidiaphragm was 0.5 to 1.0 mm longer than resting length. Preparations were stimulated with a square wave pulse at a frequency of 12 c.p.m. and a duration of 0.2 ms.)

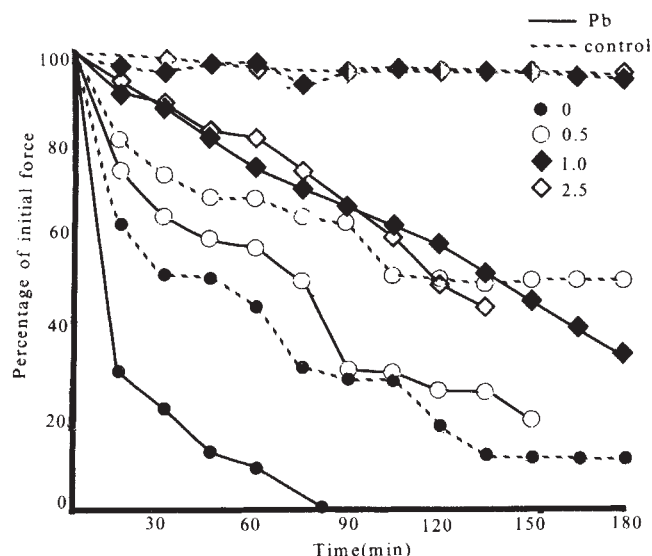


Fig. 1 Change in force of contraction (as percentage of initial force) over 3 h with varying concentrations of calcium, in lead-treated (8×10^{-5} M Pb) and control preparations. Points are means of three experiments.

All experiments were terminated after 4 h. Control preparations showed no significant decline in force of contraction nor significant changes in resting tension for the duration of the experiments.

Table 1 Effect of Lead on the Force of Contraction on Nerve and Muscle Stimulation after Treatment for 2 h

Concentration of lead ($\times 10^{-5}$ M)	n	Percentage of initial force of contraction on stimulation through:	
		Nerve	Muscle
0	15	$97.5 \pm 2.5^*$	98.0 ± 2.0
1	4	97.5 ± 2.5	98.0 ± 2.0
5	3	77.8 ± 9.7	97.5 ± 2.5
8	6	60.8 ± 5.4	97.5 ± 2.5
10	1	47.7	96.5

* Mean $\pm$ s.e.m.

Preparations exposed to lead for 2 h showed significant decreases in the force of contraction when stimulated through the nerve (Table 1). The results are expressed as percentage of the initial force of contraction for nerve and muscle stimulation of each preparation. There was no decrease in the force of contraction in controls or in preparations treated with 1×10^{-5} M lead. At 5×10^{-5} M lead, however, the force of contraction on nerve stimulation was reduced significantly to 78% of the initial force (Student's *t* test⁶). When the same preparations were stimulated through the muscle, no significant decreases in force of contraction were seen at concentrations of lead up to 1×10^{-4} M.

In a separate series of experiments, calcium was added to for preparations after exposure for 2 h to 8×10^{-5} M lead. All preparations demonstrated a decrease in nerve-stimulated force of contraction to a mean of 58% of the initial force. Calcium, when added to produce a final concentration of 3×10^{-3} M, restored the force of contraction to a mean of 91% of initial force for 28 min.

A second series of experiments was done to determine the effect of the concentration of calcium in the bathing medium on the time required for lead to affect nerve-stimulated force of contraction. The concentration of calcium was varied from 0 to 2.5 mM (isotonicity was kept constant by appropriate adjustments in the concentration of NaCl). Isometric twitch tension was measured over 3 h for controls and preparations treated with 8×10^{-5} M lead. Increasing the concentration of calcium from 0 to 1 mM significantly increases the time for equivalent impairment of nerve-

stimulated force of lead-treated preparations (Fig. 1). That is, for a given time of exposure to lead, the extent of impairment was inversely related to the calcium concentration. At calcium concentrations above 1 mM, however, no further protection against lead treatment was found.

The motor endplate was tested for possible site of lead action by the addition of exogenous acetylcholine in a range of concentrations from 1×10^{-7} to 5×10^{-6} M to the preparations after treatment for 2 h with 8×10^{-5} M lead. The contraction produced by equimolar additions of acetylcholine was identical in control and lead-treated preparations. These results tend to eliminate the motor endplate as a possible site of the primary toxic actions of lead.

The calcium experiments demonstrated that additions of calcium to normal Krebs-Ringer solution reversed the effects of treatment for 2 h with lead. Additional experiments demonstrated that the time course of impairment of neuromuscular function by lead was related to the concentration of calcium in the bathing media of the preparations. An apparent discrepancy arises between the protective and the reversing effects of calcium. Increasing the concentration of calcium from 1 to 2.5 mM did not significantly change the time course of impairment by lead. In lead-poisoned preparations, however, the addition of 0.5 mM calcium after treatment for 2 h caused a significant recovery of neuromuscular function for 28 min. The timing of calcium addition may be important in producing this reversal. These experiments indicate that calcium and lead may interact in some manner at a site important in neuromuscular function.

The experiments demonstrated that lead, in concentrations greater than 1×10^{-5} M, significantly decreased the force of contraction of the rat hemidiaphragm muscle on phrenic nerve stimulation. Over the same range of concentrations, force of contraction was not affected when the muscle was stimulated directly. Since muscle was not directly involved in the lead toxicity seen in these experiments, the effects of calcium are probably confined to junctional sites. This suggests that lead interferes with either the release of acetylcholine or the uptake of choline and subsequent synthesis of acetylcholine, processes known to have a requirement for calcium (ref. 7 and C. Pert and S. H. Snyder, personal communication).

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- ¹ Lead: *Airborne Lead in Perspective*, 71 (NAS-NRC, Washington, 1972).
- ² Reznikoff, P., and Aub, J. C., *Arch. Neurol. Psych.*, **17**, 444 (1927).
- ³ Steiman, S. E., *Am. J. Physiol.*, **125**, 261 (1939).
- ⁴ Kostial, K., and Vouk, V. B., *Br. J. Pharmacol.*, **12**, 219 (1957).
- ⁵ Bulbring, E., *Br. J. Pharmacol.*, **1**, 38 (1946).
- ⁶ Snedecor, G. W., and Cochran, W. G., *Statistical Methods*, fifth ed. (Iowa State College Press, Ames, 1956).
- ⁷ Katz, B., and Miledi, R., *J. Physiol. Lond.*, **189**, 535 (1967).

Absence of Significant Chromosome Damage in Males Occupationally Exposed to Lead

THE hazard to man of environmental contamination by certain metals is of considerable importance and in this context the organic and inorganic compounds of lead are the subject of much current interest. The clinical effects on the individual of

accidental overexposure to lead are well known. What is perhaps not so widely realised, however, is that there is the possibility that high lead levels may be hazardous to the foetus of exposed parents. "Intoxication with lead" is often quoted as "reducing fertility and increasing the frequency of abortions and miscarriages" in man¹⁻⁵, and some animal studies have shown⁶ that administration of lead salts may result in an increase in congenitally malformed offspring. These findings, the reported carcinogenicity of lead salts in rats⁷, and the earlier reports that salts of a variety of heavy metals induce aberrations in plant chromosomes^{8,9}, have stimulated a number of *in vitro* and *in vivo* studies into the possible mutagenic effects of lead which have yielded conflicting results.

Schwanitz *et al.*¹⁰ reported a three- to four-fold increase in chromosome aberration frequency in human lymphocytes treated *in vitro* with lead acetate as compared with control cells exposed to sodium acetate. In contrast, an extensive *in vitro* study on cultured Chinese hamster fibroblasts by Bauchinger and Schmid¹¹ showed no difference between control (sodium acetate) and lead acetate treated cells.

In vivo studies on mice fed on a diet containing lead acetate were reported to reveal an increased incidence of chromatid damage in cultured bone marrow cells from treated as compared with control animals. But other investigations¹² on the effect of tetraethyl lead and lead acetate in the mouse showed that neither compound, even at the maximum tolerated dose levels, promoted a dominant lethal response. In man, an increase in chromosome aberrations, particularly chromatid breaks and gaps, was reported by Schwanitz *et al.*¹⁰ to occur in the peripheral blood lymphocytes of eight male workers in a lead oxide factory.

In view of these conflicting reports our study was set up to examine the extent of chromosome damage, and any correlations between the level of damage and the lead levels in blood and urine, in a population of men occupationally exposed to oxides of lead.

The population, described in greater detail in the accompanying paper¹³, consisted of seventy male workers employed by a shipbreaking yard. Thirty-five men (Group 1) were engaged as burners to cut the hulls and all metal structures of ships, and their lengths of exposure ranged from 3 months to 43 yr. Their age range was 21-63 yr and the average weekly exposure time was 50 h. The remaining thirty-five men were of comparable age, were not actively engaged in burning, and formed a control group (Group 2).

All the men were medically examined and a full medical and occupational history taken¹³. Each worker had a periodic mass chest X ray and many had also received further radiation exposure for investigations of bone fractures and stomach ulcers. None of the men had undergone radiotherapy, and none were receiving drugs considered to produce chromosome damage. Blood samples from the men were analysed for lead content and routine haematology, and urine samples for lead and coproporphyrins¹³.

The sample of blood obtained from each individual for chromosome analysis was coded by a person not concerned in the study, before being set up in culture. The lymphocytes were cultured in Hams F10 medium with 14% bovine serum and colcemid added for the last 3 h of culture. For slide preparation, the cells were subjected to a brief hypotonic treatment with KCl¹⁴ before fixation in 3:1 (methanol-acetic acid) and preparations were made by air drying. To minimise any culture artefacts and to ensure that the majority of the dividing lymphocytes were examined at their first *in vitro* metaphase, the total culture time was either 45 or 48 h. One hundred mitoses with forty-five centromeres or more from each individual were studied by one person. The first two cells were fully analysed to ascertain the chromosome constitution of the individual and all cells were scored for aneuploidy and chromatid and chromosome aberrations.

The blood of sixty-six of the seventy workers was cultured successfully but the cultures from the four remaining individuals,

all of whom were in Group 2, yielded few mitoses of good quality and were not scored for aberrations. One of these men was found to have a 47,XXX chromosome constitution, but the remainder had normal male karyotypes.

After the scoring was completed, the slides were decoded and the cytological results obtained from the burners (Group 1) compared with those from the intended controls (Group 2). At the same time, the results from the toxicological studies were made available and from these it was apparent that a large proportion of men in the intended control group had elevated lead levels in blood and urine at the time of sampling due, presumably, to their working in the vicinity of the burners. The cytogenetic results summarised in Table 1 have therefore been divided into the four classes, A to D, based on lead levels in blood¹⁵, and further subdivided into results obtained on individuals in Groups 1 and 2 within each class.

Analysis of the data presented in the form shown in Table 1 did not reveal any consistent and significant differences between the incidence of chromosome-type and chromatid-type aberrations between Groups 1 and 2 or between classes A, B, C and D. Moreover, the results within any class were similar to those obtained from previous surveys of adult males from a variety of groups that were not considered to be unduly exposed to known or suspected mutagens (Table 1).

In considering the comparisons made between groups having different blood lead concentrations, it should be remembered that both blood and urine samples for lead estimations were obtained at the same time as blood for culture, so that these lead estimates relate only to the levels in those individuals on that particular day. The lead level in blood depends on the degree of exposure over the few days before sampling and may therefore vary considerably. On the other hand, chromosome damage in human peripheral leukocytes can reflect damage sustained many years before blood sampling (for example, in individuals exposed to ionising radiations, see below). For this reason, although we had not found any significant differences between the different blood lead-level classes, or between Groups 1 and 2, further comparisons were made within the Group 1 population. Attention was paid to the duration of exposure (number of years worked in the yard and any previous occupational history) and also to the presence of any physical or clinical (including haematological) symptoms which, however mild, could be indicative of lead poisoning. These analyses did not reveal any bias of the distribution of aberrations within the subgroups.

Although the data from the workers exposed to lead fume showed very small increases in the frequencies of chromatid breaks, and in the total number of cells with chromosome-type aberrations, the aberration frequencies were very low and were not significantly different from either control population, despite the fact that the burners, as a group, had possibly received more diagnostic X-ray exposure. There was, however, a significant difference (χ^2 (1 degree of freedom) = 31.4, $P < 0.01$) between burners and controls from the general population (but not shipyard controls) in the total number of cells which had chromatid-type aberrations. This was due to an increased incidence of chromatid and isochromatid gaps and chromatid breaks in the Group 1 men (Table 1). The scoring of these particular aberrations is extremely subjective, and whereas the cells from the Group 1 individuals were scored by a single observer, the figure derived from the 'general population' is a combination of results from several observers. These parameters of chromosome damage may be unreliable indicators due to the difficulties of distinguishing achromatic non-stained regions (gaps) and of confusing them with chromatid and isochromatid discontinuities (breaks). These can only be reliably distinguished by observing the cells at metaphase and anaphase and thus following the types of metaphase aberrations which produce fragments at anaphase. We must therefore conclude that our results do not point to an increased chromosome aberration frequency in the peripheral blood lymphocytes of individuals chronically exposed to high levels of lead oxides.

While our study was in progress, Bauchinger and Schmid¹⁶ reported on results obtained on lymphocyte chromosomes of twenty industrial workers who had been exposed for several years to lead dust, and on twenty-nine policemen "employed in the Munich areas with the highest traffic density" and who showed a 20–30% increase in blood lead levels as compared with the average population. In this work cells were cultured for 46 h and in neither of these groups was there any increase in aberration yield levels over controls.

In recording our negative findings, it is important to ask what value can be placed on the earlier reported positive result of Schwanitz *et al.*¹⁰. In that study no information is given on the duration of culture and on whether blood cultures from control and exposed individuals were set up in the same way and at the same time. The authors recorded approximately twice as many mitoses in cultures from exposed as from control individuals and if, as seems probable, the cells from the exposed individuals were cultured for 3 d, then a large proportion of the metaphase cells may have been in their second or later mitosis in culture^{16,17}. It is well known, from studies on normal and irradiated human blood cells, that the incidence of gaps and chromatid aberrations in lymphocyte chromosomes is increased if cells are allowed to proceed through two or more cycles in culture, as also is the incidence of polyploid mitotic figures and of "derived" chromosome-type changes¹⁸. The fact that Schwanitz *et al.*¹⁰ observed some eighteen tetraploid metaphases in a total of 800 scored in the exposed individuals (compare the 0 in 3,500 scored in the present work) and that the excess aberrations in this group were "secondary aberrations", that is, largely chromatid and isochromatid gaps, suggests that most of these aberrations were not present in these cells *in vivo*, but developed during culture. This suggestion raises the very important general question of interpretation in the case of chromosome damage in lymphocytes obtained from individuals who may or may not have been exposed to an environmental mutagen.

Many of the lymphocytes in human peripheral blood are

long lived and a significant proportion rarely undergo mitosis *in vivo*¹⁹. Chromosome damage sustained by these cells at the time of their exposure to a mutagen may therefore be detectable for many years after exposure, as in the case of the Japanese A-bomb survivors who show high aberration yields some 25 yr after their original exposure²⁰. Circulating lymphocytes are in a G₁ state so that exposure to ionising radiations induces chromosome-type changes where the unit of breakage and exchange is the whole chromosome giving the characteristic dicentric, rings and chromosome fragments. Irradiation of the S or G₂ phases of the cell cycle gives chromatid-type aberrations where the unit of breakage or exchange is the single chromatid. In contrast with ionising radiations, many chemicals produce only chromatid-type aberrations in the first and subsequent mitosis following exposure, even though the cells may have been exposed to the mutagen while in the G₁ phase of the cycle¹⁸. The less frequent chromosome-type changes seen in second, or later, divisions following treatment with these chemical mutagens result from the duplication of chromatid aberrations that were transmitted to daughter cells, and are thus 'derived' chromosome changes¹⁸.

In the case of chronic *in vivo* exposure to a chemical mutagen, one might expect to observe both chromatid and derived chromosome damage in lymphocytes at their first mitosis in culture. The incidence of such damage will, however, be dependent not only on the accumulated dose, but also on the interval between exposure and time of sampling for culture. With increasing time there would be an increasing probability of obtaining derived chromosome changes due to cell turnover *in vivo*, but a corresponding reduction in the incidence of chromatid-type changes due to cell, and aberration, loss and possibly also to repair of lesions in G₁ chromosomes. Chromatid damage observed at the first mitosis in culture will therefore be heavily influenced by the amount of mutagen present in the body over a relatively short time before sampling and also by the amount of mutagen inevitably carried over in the blood into the culture medium.

Table 1 Cytogenetic Findings in Cultured Lymphocytes from Shipyard Workers with an Occupational History of Lead Exposure (Group 1), and from Shipyard Controls (Group 2) and Other Adult Males

Category	Blood lead level (µg per 100 ml)	No. of men studied	Total cells scored	% Normal cells	Cells with chromatid aberrations					Cells with chromosome aberrations				
					Total No.	%	Chromatid and isochromatid gaps	Chromatid breaks	Chromatid interchanges	Total No.	%	Dicentric	Acentric fragments	No. structurally abnormal chromosomes*
A. Normal	<40	12 (Group 2)	1,200	91.5	51	4.25	53	0	0	5	0.42	2	5	0
B. Acceptable	40–80	16 (Group 1)	1,600	93.33	71	4.43	68	7	2	7	0.44	1	6	5
		17 (Group 2)	1,700	94.50	70	4.12	73	1	2	6	0.35	0	5	2
		33 (total)	3,300	93.47	141	4.27	141	8	4	13	0.39	1	11	7
C. Excessive	80–120	15 (Group 1)	1,500	93.87	72	4.80	68	5	0	13	0.87	0	7	3
		2 (Group 2)	200	94.29	10	5.00	10	1	0	1	0.50	0	1	0
		17 (total)	1,700	94.09	82	4.90	78	6	0	14	0.82	0	8	3
D. Dangerous	>120	4 (Group 1)	400	94.50	25	6.25	25	0	0	3	0.75	0	3	0
Total Group 1 (Exposed)	—	35	3,500	93.90	168	5.16	161	12	2	23	0.69	1	16	8
Total Group 2 (Control)	—	31	3,100	93.43	131	4.46	136	2	2	12	0.42	2	11	2
Other males†	—	285	3,107	96.66‡	68	2.18	65	4	0	36	1.16	2	9	16

* Number of structurally abnormal monocentric chromosomes observed which were not associated with an acentric fragment.

† Data obtained from previous MRC surveys of other adult male groups with no history of undue exposure to known or suspected mutagens (lymphocyte culture time <50 h).

‡ Includes non-modal cells.

In terms of potential genetic hazard to a population, the production of a low level of damage in a large number of individuals is of greater consequence to the population than the induction of large amounts of damage in a small number of individuals. The human peripheral blood lymphocyte offers a convenient means of detecting low levels of chromosome damage which might be caused by an environmental agent and may provide a useful screening system. But, apart from the considerable technical labour involved, there may be difficulties both with the interpretation of chromosome aberration data and with the extrapolation of results from somatic to germ cells. Problems of interpretation are further complicated by mutagens which produce chromatid-type aberrations, since similar changes may also develop in cultured cells as a consequence of infection or of non-specific 'adverse culture conditions'. In such work it is obviously of paramount importance to use optimum culture conditions and to obtain proper control specimens matched not only in terms of donor characteristics but also with regard to culture conditions.

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- ¹ Muro, L. A., and Goyer, R. A., *Archs Path.*, **87**, 660 (1969).
- ² Weller, C. V., *J. med. Res.*, **33**, 271 (1915).
- ³ Potter, E. L., in *Pathology of the Fetus and Newborn*, 168 (Year Book Medical Publishers, Chicago, 1961).
- ⁴ Gillet, J. A., *Lancet*, **i**, 1118 (1955).
- ⁵ Nishimura, H., in *Chemistry and Prevention of Congenital Abnormalities*, 13 (Thomas, Springfield, Illinois, 1964).
- ⁶ Ferm, V. H., and Carpenter, S. J., *Expl. molec. Path.*, **7**, 208 (1967).
- ⁷ Hass, G. M., McDonald, J. H., Oyasu, R., Battifora, H. A., and Paloucek, J. T., in *Renal Neoplasia* (edit. by King, J. S., jun.) (Little, Brown, Boston, 1967).
- ⁸ Glass, E., *Zeit Bot.*, **43**, 359 (1955).
- ⁹ Steinman, I. D., Venkatram, N. I., and Szybalski, W., *Archs Biochem. Biophys.*, **76**, 78 (1958).
- ¹⁰ Schwanitz, G., Lehnert, G., and Gebhart, E., *Deut. Med. Wochschr.* (English Language Edition), **15**, 738 (1970).
- ¹¹ Bauchinger, M., and Schmid, E., *Mutat. Res.*, **14**, 95 (1972).
- ¹² Kennedy, G. L., and Arnold, D. W., *Newsl. Environ. mutag. Soc.*, **5**, 37 (1971).
- ¹³ Taylor, W., Molyneux, M. K. B., and Blackadder, E. S., *Nature*, **247**, 53 (1974).
- ¹⁴ Hungerford, D. A., *Stain Technol.*, **40**, 333 (1965).
- ¹⁵ Bauchinger, M., Schmid, E., and Schmidt, D., *Mutat. Res.*, **16**, 407 (1972).
- ¹⁶ Buckton, K. E., and Pike, M. C., *Int. J. rad. Biol.*, **8**, 439 (1964).
- ¹⁷ Sasaki, M. S., and Norman, A., *Nature*, **210**, 913 (1966).
- ¹⁸ Evans, H. J., in *Human Population Cytogenetics* (edit. by Jacobs, P. A., Price, W. H., and Law, P.), 191 (Edinburgh University, Edinburgh, 1970).
- ¹⁹ Buckton, K. E., Smith, P. G., and Court Brown, W. M., in *Human Radiation Cytogenetics* (edit. by Evans, H. J., Court Brown, W. M., and McLean, A. S.), 106 (North-Holland, Amsterdam, 1967).
- ²⁰ Report of United Nations Scientific Committee on the Effects of Atomic Radiation, UN General Assembly 24th Session Supplement 13 (A/7613) (1969).

per 100 ml of whole blood. In this operation, in which oxy-gas torches have been used at the work face, temperatures are in the region of 3,000–3,500° C. Iron melts at 1,535° C and is found in vapour form at 3,000° C. Lead melts at 328° C and boils at 1,740° C. This produces a risk of excessive exposure in compartments and between decks of ships, of which lead from paintwork is the principal risk³. In the breathing zone of the burner, with maximum concentrations of fume at torch level, may also be found zinc (zinc metal fume fever), copper from piping and cadmium from protective coatings⁴.

The limitation of statutory control measures and the difficulty of clinically detecting lead absorption (as opposed to lead poisoning) have led to ineffective control in shipbreaking processes compared with other industries with a known lead risk (as, for example, in lead accumulator manufacture). The evaluation of the degree of lead absorption and the identification of lead overexposure is clearly the right objective in modern conditions, rather than the identification of lead poisoning⁵. It is not yet known what levels of lead absorption are tolerable but Lane *et al.*⁶ have proposed four arbitrary categories, (A) absorption found in the 'normal' population (blood lead <40 µg per 100 ml; urinary lead <80 µg l⁻¹); (B) increased but acceptable absorption (blood lead 40–80 µg per 100 ml; urinary lead 80–150 µg l⁻¹); (C) excessive and unacceptable absorption (blood lead 80–120 µg per 100 ml; urinary lead 150–250 µg l⁻¹); and (D) dangerous absorption, suspend automatically from work with lead (blood lead >120 µg per 100 ml; urinary lead >250 µg l⁻¹).

This paper is concerned with the examination of a population of oxy-gas burners in a shipbreaking yard exposed to lead fume in their work environment. In the accompanying paper⁷ cytogenetic studies on this population are described.

In one shipbreaking yard thirty-five males engaged on oxy-gas burning (Group 1) and a control group of thirty-five men of comparable age employed as labourers, crane drivers, and so on (Group 2) were investigated. The age distribution of the population and the length of time the oxy-gas burners had been actively burning are shown in Tables 1 and 2.

Table 1 Ages of Burners and Controls in Ten-yr Age Groups

Age (yr)	No. of burners, ship and shore (Group 1)	No. of controls (Group 2)
15–24	6	6
25–34	11	3
35–44	2	9
45–54	8	9
55–64	8	8
Total	35 (Mean age 41 yr)	35 (Mean age 43 yr)

Table 2 Length of Time Employees (Group 1) had been Burners

Oxy-gas burning (yr)	Number of Men	%
Up to 5 yr	20	57
5 yr and over	15*	43
Mean 8.8	35	100

* Two of these men had 42 and 43 yr continuous burning.

Lead Over-absorption in a Population of Oxy-gas Burners

PLINY (AD 23–79) observed that in the second century BC the ancients painted their ships with native ceruse (white lead), that lead poisoning was known in his day and that the workers in lead products tied up their faces in loose bags lest they should inhale the pernicious dust¹. In a survey of 228 men in nine shipbreaking yards in Scotland and NE England, McCallum² focused attention on the health hazard resulting from burning lead-painted metal with a high temperature flame. He found 36% of the burners with a haemoglobin content less than 12.5 g

A full occupational and medical history, including previous spells of burning at other yards, was obtained for burners and controls, with emphasis on the symptoms of lead absorption, which are colic, dyspepsia, lassitude and loss of appetite, constipation, muscle aches and headache. The physical signs of lead over-absorption, such as paresis, pallor and blue line on the gums, were then looked for. In the first survey a sample (20 ml) of venous blood was taken from each man and divided into two equal parts, one being used for lymphocyte culture for chromosome analysis and the other for blood lead content and

haematological studies. In addition, urinary lead determinations were carried out by the Central Reference Laboratory of the Department of Employment. In order to obtain a more accurate picture of the extent of lead absorption of this population previous to and at the time of the cytogenetic study, four additional burner population surveys for blood and urine lead were made at three-monthly intervals. An environmental survey was carried out by the Industrial Hygiene Laboratory of HM Factory Inspectorate. Samples were taken from the breathing zone of burners cutting heavily painted panels of a merchant ship and of a naval frigate.

The mean figures obtained for the burners and controls from the first survey for lead levels in blood and urine are shown in Table 3, together with the positive findings of the clinical examination. It should be noted that the thirty-five burners sampled gave a mean haemoglobin value of 14.8 g per 100 ml with seven under 14.0 g and one under 12.5 g per 100 ml.

Table 3 Survey 1. Lead Levels of Blood and Urine, and Clinical Symptoms

Population	Blood lead (μg per 100 ml)	Urine lead ($\mu\text{g l}^{-1}$)	Colic	Constipation	Pallor	Lassitude	Blue line
Group 1*	mean=91.0 (35 men)	median=144 (32 men)	4	3	4	4	5
Group 2†	mean=49.7 (34 men)	median=24 (32 men)	-	-	-	-	-

* Oxy-gas burners.

† Controls.

The difference in mean blood lead levels between Group 1 and the Group 2 controls is significant at the 0.1% level. A number of urine lead estimations were below the lower limit of detection of the chemical method, so that the urine lead results were analysed by non-parametric tests where medians were used instead of means. The difference between the oxy-gas burners and the control population was once again significant at the 0.1% level. The detailed data, together with the results from subsequent surveys on the burner population, are summarised according to the classification of Lane *et al.*⁶ in Tables 4 and 5.

Table 4 Lead Absorption Categories⁶ in Blood in the Four Surveys of a Population of Oxy-gas Burners

Survey	Date	Absorption category (μg per 100 ml)				Total No. of burners
		A Normal (<40)	B Accept- able (40- 80)	C Exces- sive (80- 120)	D Dan- gerous (>120)	
1	November 1971	14	19	2	0	35 controls
1	November 1971	0	16	15	4	35
2	April 1972	0	9	17	12	38
3	July 1972	3	24	7	0	34
4	November 1972	12	24	2	0	38

The results of the environmental survey showed that from heavily painted plates of a merchant ship and hull sections of a warship, concentrations approaching 4.0 mg and 14.0 mg m^{-3} lead-in-air respectively were produced in the breathing zones. These values are very much higher than the threshold limit value for a 40 h week of 0.2 mg m^{-3} (ref. 8). There is, however, no accurate method of computing actual lead intake from concentrations of lead-in-air which vary over very wide limits dependent on the presence or absence of lead paint, constantly varying air movements, and variable absorption due to voluntary use of respirators.

Table 5 Lead Absorption Categories⁶ in Urine in the Four Surveys of a Population of Oxy-gas Burners

Survey	Date	Absorption category ($\mu\text{g l}^{-1}$)				Total No. of burners
		A Normal (<80)	B Accept- able (80- 150)	C Exces- sive (150- 250)	D Dan- gerous (>250)	
1	November 1971	28	5	0	0	33* controls
1	November 1971	6	11	10	7	34*
2	April 1972	10	16	6	6	38
3	July 1972	15	15	4	0	34
4	November 1972	23	12	3	0	38

* Excluding samples that were contaminated and not recorded.

The results of biological tests on the thirty-five oxy-gas burners in this study confirm the views of McCallum^{2,3}. In our initial survey, high blood lead levels were found in some of the selected controls, but all individuals in Group 1 had more than 40 μg of lead per 100 ml of blood, with four men in the dangerous (D) category (Table 4). In the second survey carried out three months later, a greater proportion of the exposed population had higher blood lead levels, with twelve men in the D category. These burners with the highest blood lead levels were then removed from burning where there was any risk of lead in the fume and subsequent surveys (Table 4) showed a marked reduction in blood lead levels. The urine lead values (Table 5), while not correlating with the blood values, showed the same degree of improvement over one year's monitoring. The data obtained in these surveys show that by close supervision of the work situation, it is possible to achieve a degree of lead absorption which is acceptable by today's arbitrary standards.

A number of questions remain unanswered in the present shipbreaking work environment. One is the permanence or long-term medical effects of lead absorption in which Rieke⁹ has recently expressed concern, particularly with regard to the detection of so-called 'susceptible' burners. A second is the possibility of chromosome damage and this is the subject of the accompanying paper⁷.

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¹ Hunter, D., *The Diseases of Occupations* (English Universities Press, London, 1955).

² McCallum, R. I., Sanderson, J. T., and Richards, A. E., *Ann. occup. Hyg.*, **11**, 101 (1968).

³ McCallum, R. I., *Ann. occup. Hyg.*, **6**, 55 (1963).

⁴ Evans, D. M., *Br. med. J.*, **1**, 173 (1960).

⁵ Windeyer, B. S., *Report of a Committee Appointed to Inquire into Lead Poisonings at the RTZ Smelter at Avonmouth* (HMSO, London, Cwmnd. 5042, 1972).

⁶ Lane, R. E., *Br. med. J.*, **4**, 501 (1968).

⁷ O'Riordan, M. L., and Evans, H. J., *Nature*, **247**, 50 (1974).

⁸ *Documentation of the Threshold Limit Values* (American Conference of Governmental Industrial Hygienists, Cincinnati, 1971).

⁹ Rieke, F. E., *Archs envir. Hlth*, **19**, 521 (1969).

Clonal Restriction of the Immune Response to Phosphorylcholine

THE antibody response to most antigens is chemically heterogeneous, reflecting clonal heterogeneity of the responding cells. This heterogeneity may arise in part from the number of different epitopes present in the structure of complex antigens, although the antibody response even to chemically simple haptens (which may contain more than one epitope) is often very heterogeneous. For example, the dinitrophenyl hapten on a protein carrier elicits many different anti-hapten antibodies which are distinguished by isoelectric focusing¹. Also, individual mice respond to the *p*-azobenzoate and to the *p*-azophenylarsonate haptens by producing a population of serum antibodies with many idiotypic specificities². Certain carbohydrate antigens, however, seem to induce relatively homogeneous antibody responses^{3,4}. One of these antigens, the C-polysaccharide from the rough strain of pneumococcus R36A, induces a restricted antibody response directed against phosphorylcholine⁵, a structural component of the C-polysaccharide⁶.

We have examined the antibody response of Balb/c mice to phosphorylcholine by testing various inhibitors for their capacity to interfere specifically with antibody activity. These inhibitors include (a) phosphorylcholine and phosphorylcholine analogues to determine the epitopic specificity of the antibodies and (b) antisera raised against myeloma proteins which have antibody activity against phosphorylcholine. These anti-idiotypic sera provide a measure of the idiotypic, and thus clonal homogeneity of the response. By these methods we find that the antibody response is remarkably homogeneous and has the characteristics of a so-called "monoclonal" response. In fact, the antibody response is equally homogeneous whether immunisation is with the C-polysaccharide or with phosphorylcholine coupled to various protein carriers. Furthermore, when this response is suppressed, other clones of cells producing antibody to phosphorylcholine do not appear.

p-Diazophenylphosphorylcholine was synthesised according to the method of Chesebro and Metzger⁷. This compound, in pH 9.2 borate buffer, reacts with tyrosyl residues of proteins to yield the conjugated hapten, *p*-azophenylphosphorylcholine (PC). Balb/c mice were immunised with PC conjugated to keyhole limpet haemocyanin (PC-KLH) or PC conjugated to *Salmonella* flagella (PC-FLA). The synthesis of antibodies by individual spleen cells was demonstrated by the haemolytic plaque technique, using as target cells sheep erythrocytes conjugated with PC (PC-SRBC)⁵.

Table 1 Response to *p*-Azophenylphosphorylcholine Conjugated to Different Carriers

Antigen	Carrier priming	PFC per spleen $\times 10^3$
10 ⁹ cells R36A	—	78.4 $\pm$ 6.3
200 μ g FLA	—	2.3 $\pm$ 1.2
200 μ g PC-FLA	—	287.3 $\pm$ 27.0
20 μ g PC-FLA	—	162.0 $\pm$ 7.2
200 μ g KLH	—	0.9 $\pm$ 0.2
200 μ g PC-KLH	—	151.0 $\pm$ 18.0
20 μ g PC-KLH	—	95.0 $\pm$ 13.0
200 μ g PC-FLA	5 μ g FLA	269.0 $\pm$ 20.8
20 μ g PC-FLA	5 μ g FLA	148.3 $\pm$ 16.0
200 μ g KLH	5 μ g KLH	1.8 $\pm$ 0.6
200 μ g PC-KLH	5 μ g KLH	439.8 $\pm$ 52.4
20 μ g PC-KLH	5 μ g KLH	328.4 $\pm$ 24.3

8–12-week-old Balb/c mice were immunised intravenously with antigen 4 d before being assayed for their splenic PFC response. PC-KLH and PC-FLA were in saline solution while the R36A vaccine was a saline suspension of the formalin-killed bacteria⁵.

The magnitude of the IgM plaque-forming cell response is given in Table 1; about 8×10^4 plaque-forming cells (PFC) per spleen were detected in mice immunised with the R36A pneumococcal vaccine while immunisation with either 200

μ g of PC-KLH or of PC-FLA induced about 15×10^4 and 30×10^4 PFC per spleen respectively. Immunisation with either carrier alone caused an insignificant response to PC. Thus, the hapten on either carrier elicited a splenic PFC response slightly greater than that induced by the vaccine. When mice were primed with a small dose of KLH 3 d before immunisation with PC-KLH, the anti-hapten response was about three times greater than the response obtained from unprimed mice. No such carrier priming effect was observed with the PC-FLA antigen.

While the effect of carrier priming was demonstrable for PC-KLH, the effect of priming is even more pronounced for other carriers. For example, PC coupled to bovine gamma globulin (BGG) induces a very low response in Balb/c mice; however, the response is comparable with that reported here when mice are primed with BGG (W. L., unpublished results). Thus, carrier-specific cells are required, at least for some PC-carrier conjugates; and, by inference, the magnitude of responses to these conjugates is a function of the number of carrier-specific cells present.

Indirect plaques were not detected during the primary or the secondary response to PC induced by immunisation with either PC-KLH or PC-FLA. The developing serum used for enhancement of IgG PFC was a goat anti-mouse IgG serum which was effective for demonstrating indirect plaques against sheep red blood cells (SRBC)⁸. Apparently, the response is restricted to production of IgM antibodies.

The specificity of the PFC response to PC was studied using analogues of *p*-azophenylphosphorylcholine to inhibit plaque formation by spleen cells from mice immunised with different PC-carrier conjugates; 10^{-2} mg ml⁻¹ PC-BSA or 10^{-2} M phosphorylcholine solutions inhibited formation of plaques. In contrast, choline alone, acetylcholine alone or 2-dimethylaminoethylphosphate alone, at the same molarity, failed to inhibit plaque formation, Table 2a. Thus, it seems that the intact zwitterionic phosphorylcholine molecule is required to inhibit the binding of anti-hapten antibodies to the PC-SRBC. None of the above compounds inhibited plaque formation by spleen cells from mice immunised with

Table 2 Inhibition of Plaque Formation

Inhibitors	% Inhibition of anti-hapten plaques from mice immunised with			
	PC-FLA	PC-KLH	PC-KLH (KLH-primed)	TNP-KLH
(a) Hapten analogues				
—	0 $\pm$ 3 %	0 $\pm$ 4 %	0 $\pm$ 2 %	0 $\pm$ 4 %
10 ⁻² M phosphorylcholine	98 $\pm$ 2 %	98 $\pm$ 3 %	99 $\pm$ 2 %	4 $\pm$ 3 %
10 ⁻² mg ml ⁻¹ PC-BSA	97 $\pm$ 2 %	97 $\pm$ 2 %	97 $\pm$ 3 %	2 $\pm$ 4 %
10 ⁻² M choline	0 $\pm$ 2 %	2 $\pm$ 4 %	4 $\pm$ 3 %	0 $\pm$ 4 %
10 ⁻² M acetylcholine	1 $\pm$ 4 %	1 $\pm$ 4 %	4 $\pm$ 2 %	0 $\pm$ 4 %
10 ⁻² M 2-dimethylaminoethyl phosphate	1 $\pm$ 4 %	3 $\pm$ 3 %	2 $\pm$ 3 %	2 $\pm$ 7 %
10 ⁻² mg ml ⁻¹ BSA	-6 $\pm$ 5 %	-2 $\pm$ 4 %	0 $\pm$ 4 %	2 $\pm$ 5 %
(b) Anti-idiotypic sera				
anti-TEPC-15	97 $\pm$ 3 %	99 $\pm$ 3 %	96 $\pm$ 2 %	-2 $\pm$ 4 %
anti-MOPC-167	-2 $\pm$ 3 %	-7 $\pm$ 5 %	0 $\pm$ 4 %	-5 $\pm$ 4 %
anti-McPC-603	5 $\pm$ 3 %	4 $\pm$ 4 %	7 $\pm$ 4 %	2 $\pm$ 4 %

The anti-hapten PFC were from Balb/c mice immunised intravenously with antigen 4 d before assay. Inhibition of plaque formation was carried out as described previously⁵. The effect of each inhibitor was assayed in triplicate. Inhibition is presented as the % inhibition of plaques $\pm$ s.e. by each agent. Anti-TNP plaques were used to test for the non-specific inhibitory activity of the agents used. Anti-TNP PFC were assayed using TNP-SRBC as target cells⁹. The anti-idiotypic sera used for plaque inhibition were absorbed with normal Balb/c serum, heat-inactivated and diluted 1:100 in Hanks balanced saline solution before use in this assay. The concentration of phosphorylcholine required for 50% inhibition is the same for PFC from PC-FLA, PC-KLH or R36A immunisation. Furthermore, the inhibition curve for plaques from animals immunised with PC-KLH indicates homogeneity of binding affinities among the PFC (W. L., unpublished results).

the trinitrophenyl hapten conjugated to KLH (TNP-KLH) and assayed against TNP-SRBC⁹. From these findings, we conclude that the anti-PC response measured by this assay is directed to phosphorylcholine itself and not to determinants introduced by the conjugation of phosphorylcholine to carriers.

The idiotypic homogeneity of the response to the PC hapten was studied by the plaque inhibition technique⁵ using antisera raised against the myeloma proteins TEPC-15, MOPC-167 and McPC-603. Although these Balb/c myeloma proteins bind phosphorylcholine, each possesses a different idio-¹⁰. The antisera, raised in A/He mice^{5,10} to these three proteins, were used to determine the idiotypic specificity of the PFC response to PC, Table 2b. Anti-MOPC-167 and anti-McPC-603 had no inhibitory effect while anti-TEPC-15 inhibited plaque formation by cells from mice immunised with either of the PC-carrier conjugates. None of the antisera inhibited plaque formation to the TNP hapten. Thus, the response to phosphorylcholine is of an idio-¹¹ type identical to or cross reactive with the idio-¹² type of the TEPC-15 myeloma protein.

Mice given anti-TEPC-15 serum before immunisation have reduced responses to phosphorylcholine¹¹. Suppression is due to inhibition of immunisation rather than to inhibition of plaque formation, since no suppression occurs when the antiserum is given 1 d after immunisation¹¹. In our experiments, anti-TEPC-15 sera were administered to Balb/c mice 1 d before immunisation with PC-carrier, Table 3. The response of these mice, after immunisation with PC-FLA or PC-KLH, was less than 5% of the response of normal mice immunised with the same antigen. The idiotypic specificity of suppression was demonstrated by the failure of anti-MOPC-167 or of anti-McPC-603 to suppress the responses induced by PC-KLH. That the suppression was specific for the PC hapten was shown by the inability of anti-TEPC-15 serum to diminish the response to the TNP hapten. In fact, when mice were immunised with both PC and TNP conjugated to the same carrier molecule (PC-KLH-TNP), anti-TEPC-15 serum suppressed only the response to PC, Table 3; obviously carrier-specific helper cell activity for TNP is not affected by suppression of the response to PC.

Table 3 Specific Suppression of the Response to Phosphorylcholine by Anti-idiotypic Serum

Antigen	Antiserum	PFC per spleen $\times 10^3$	TNP
50 μ g PC-FLA	—	112.9 $\pm$ 12.1	
50 μ g PC-FLA	anti-TEPC-15	1.6 $\pm$ 0.9	
200 μ g PC-KLH	—	140.5 $\pm$ 11.7	
200 μ g PC-KLH	anti-TEPC-15	3.1 $\pm$ 1.3	
200 μ g PC-KLH	anti-MOPC-167	136.3 $\pm$ 8.2	
200 μ g PC-KLH	anti-McPC-603	154.2 $\pm$ 13.5	
200 μ g TNP-KLH	—	283.9 $\pm$ 15.4	
200 μ g TNP-KLH	anti-TEPC-15	294.0 $\pm$ 23.9	
200 μ g PC-KLH-TNP	—	99.6 $\pm$ 12.9	120.3 $\pm$ 4.0
200 μ g PC-KLH-TNP	anti-TEPC-15	1.9 $\pm$ 1.3	106.8 $\pm$ 10.3

Balb/c mice treated with anti-idiotypic sera were given 0.3 ml of the appropriate antisera intravenously 1 d before immunisation with antigen, and their spleens assayed for anti-hapten PFC 4 d after immunisation. Anti-PC PFC were assayed using PC-SRBC as described in Table 1. Anti-TNP PFC were assayed using TNP-SRBC⁹. The anti-hapten PFC per spleen are the mean from groups of three mice $\pm$ s.e.m.

Whereas suppression of induction of the response to PC by antibody to TEPC-15 is considerable, it is not complete. The PFC still detectable in suppressed mice were analysed to determine whether the antibodies secreted by these cells were directed to phosphorylcholine and if so whether they bear the TEPC-15 idio-¹³. The results, Table 4, indicate that about 50% of these residual plaques are no longer inhibited by free phosphorylcholine. Of the plaques that are inhibitable by phosphorylcholine, apparently very few, if any, are of the TEPC-15, MOPC-167 or McPC-603 idio-

types, since the antisera to these myeloma proteins did not significantly inhibit the residual plaques. Thus, the epitopic specificity and the idiotypic composition of the residual response after suppression with anti-idiotypic serum to TEPC-15 are different from the normal anti-PC response.

We have shown that Balb/c mice respond as well to phosphorylcholine whether they are immunised with the R36A pneumococcal vaccine or with *p*-azophenylphosphorylcholine-carrier conjugates. The inhibition studies demonstrate that the measured response to *p*-azophenylphosphorylcholine is directed to the phosphorylcholine moiety of the hapten. The response to phosphorylcholine is also restricted to the idio-¹⁴ present in the TEPC-15 myeloma protein regardless of the carrier used to induce the antibody response. Furthermore, priming with carrier does not affect this idio-¹⁵ restriction. These findings lead us to conclude that the same B cell clone or clones having receptors for phosphorylcholine are stimulated regardless of the carrier used. Apparently the carrier has no effect on the selection of the B cell clone responding to PC.

Table 4 Analysis of the PFC Remaining after Suppression by Anti-idiotypic Serum

Inhibitor	% Inhibition of anti-PC plaques from mice immunised with	
	PC-FLA	PC-KLH (KLH-primed)
—	0 $\pm$ 5%	0 $\pm$ 7%
10 ⁻² M phosphorylcholine	54 $\pm$ 7%	45 $\pm$ 9%
10 ⁻² mg ml ⁻¹ PC-BSA	96 $\pm$ 4%	95 $\pm$ 6%
anti-TEPC-15	7 $\pm$ 4%	6 $\pm$ 6%
anti-MOPC-167	-4 $\pm$ 7%	-4 $\pm$ 8%
anti-McPC-603	13 $\pm$ 6%	1 $\pm$ 10%

The plaques studied here were from animals suppressed with anti-TEPC-15 and immunised with 200 μ g of PC-carrier intravenously 1 d later. PC-KLH-immunised mice had been primed with 5 μ g KHL 3 d before immunisation. The assay technique and the statistical analysis were the same as those used for the data in Table 2.

The residual response in suppressed mice is not of the TEPC-15 idio-¹⁶. Furthermore, this response may involve the chemical bridge between PC and the carrier, since plaque formation is suppressed by PC-carrier but not by phosphorylcholine alone. Whether this residual response is present as a minute and hence undetectable fraction of the normal response, or whether it arises only after suppression of the dominant TEPC-15 idio-¹⁷, remains undetermined.

The possibility existed that, on immunisation of mice with PC, expression of the dominant TEPC-15 idio-¹⁸ might be responsible for suppressing the expression of other idio-¹⁹. For example, in the heterogeneous responses to haptens such as DNP and *p*-azophenylarsonate, the loss of expression of one idio-²⁰ or clone is followed by compensatory activation of other clones, some of which were previously silent^{12,13}. When the clone or clones of cells expressing the TEPC-15 idio-²¹ are suppressed, however, other clones fail to expand appreciably, if at all. This makes it unlikely that expression of the dominant TEPC-15 clone is actively suppressing the other clones. Furthermore, mice suppressed with anti-TEPC-15, and then challenged several times with PC-carrier until a PFC response to PC is again detected, still expressed only the TEPC-15 idio-²² (unpublished results of W. L.). This recovery from suppression with expression of the suppressed idio-²³ strongly suggests that the response of Balb/c mice to PC is restricted to the TEPC-15 idio-²⁴. Presumably, this restriction in the adult Balb/c mouse is due either to clonal selection during ontogeny or to the absence or genetic deficiency of other clones. The study of the response to phosphorylcholine in mice which have been idiotypically suppressed at different stages of ontogeny may indicate the origin of this clonal restriction. In addition, the natural occurrence of a response which is restricted to the expression of one dominant idio-

type may provide an experimental system for the study of the senescence of a repeatedly stimulated clone in the same animal. These possibilities are under investigation.

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- ¹ Williamson, A. R., *Eur. J. Immun.*, **1**, 390 (1971).
- ² Hart, D. A., Wang, A. I., Pawlak, L., and Nisonoff, A., *J. exp. Med.*, **135**, 1293 (1972).
- ³ Eichmann, K., *Eur. J. Immun.*, **2**, 301 (1972).
- ⁴ Briles, D. E., and Krause, R. M., *J. Immun.*, **109**, 1311 (1972).
- ⁵ Cosenza, H., and Köhler, H., *Science, N.Y.*, **176**, 1027 (1972).
- ⁶ Tomasz, A., *Science*, **157**, 694 (1968).
- ⁷ Chesebro, B., and Metzger, H., *Biochemistry*, **11**, 766 (1972).
- ⁸ Cosenza, H., and Nordin, A. A., *J. Immun.*, **104**, 976 (1970).
- ⁹ Rittenberg, M. B., and Pratt, *Proc. Soc. exp. Biol. Med.*, **132**, 572 (1969).
- ¹⁰ Potter, M., and Libermann, R., *J. exp. Med.*, **132**, 737 (1970).
- ¹¹ Cosenza, H., and Köhler, H., *Proc. natn. Acad. Sci., U.S.A.*, **69**, 2701 (1972).
- ¹² Askonas, B. A., and Williamson, A. R., *Nature*, **238**, 339 (1972).
- ¹³ Hart, D. A., Pawlak, L., and Nisonoff, A., *Eur. J. Immun.*, **3**, 44 (1973).

Lacunae in Erythrocyte Monolayers induced by Adherent Leukocytes

POLYMPHONUCLEAR leukocytes are capable of migration, aggregation, adherence, and phagocytosis as well as the intracellular destruction of bacteria¹. The observations reported here suggest yet another facility, namely that of repulsion. These experiments indicate that surface adherent leukocytes, whether polymorphonuclear or other types, repel erythrocytes from the immediate zone about them, thus creating lacunae.

Buffy coat fractions were obtained from fresh heparinised human blood by centrifugation in plastic tubing². These were mixed in a vortex shaker with an equal aliquot of red cells diluted ten-fold in 0.9% sodium chloride solution. Ten microlitres of this was added to a siliconised glass slide, covered with an 18 mm circular coverslip, and the edges sealed with paraffin oil. After incubation at 37.5° C for between 30 min and 4 h, the slides were viewed at varying intervals over several days, magnification of 150 to 450, with ordinary phase, interference phase and darkfield phase illumination.

The usual preparation was a uniform monolayer of erythrocytes with a variable number of distinct, round, clear lacunae. In some instances, twin lacunae were seen, and in particularly thin preparations the "lake" pattern could be distorted. These lacunae in the erythrocytes developed early, increased in number and size on standing overnight, and persisted for days. Many were near the edge of the preparation, but they were also seen scattered uniformly throughout. In thick preparations, the lacunae appeared only as light areas in the diffuse redness of multilayered erythrocytes.

Most of the lacunae were acellular except for a single central cell identifiable as a polymorphonuclear leukocyte (Fig. 1), or occasionally as a monocyte, basophil, or lymphocyte. Occasionally, a few platelets or one or two red cells could be seen intruding into the lacuna. A few

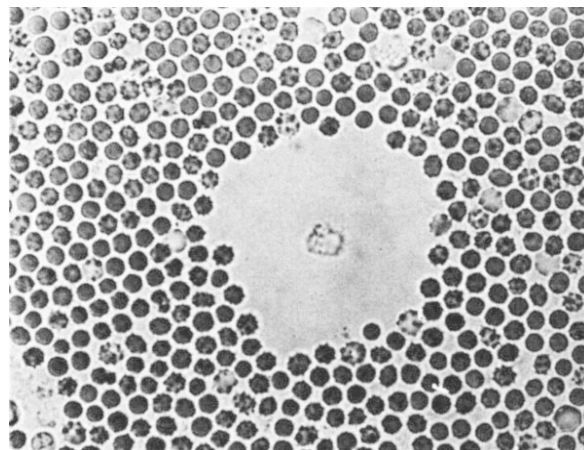


Fig. 1 View of lacuna which appears around an adherent polymorphonuclear leukocyte. In lower part of field, a lymphocyte floats freely in the erythrocytes, failing to show lacuna formation. Unstained, $\times 419$.

lacunae were completely devoid of even a central cell. Under phase microscopy³ many lymphocytes could be identified among the erythrocytes, without any circum-cellular space or lacunar formation. Similarly, platelet clumps were seen among the erythrocytes but again without any surrounding clear spaces.

The phenomenon of lacuna formation seems not to be limited to human blood, being similarly observed in rabbit, rat and mouse blood. It occurred on plain glass and plastic, as well as on siliconised glass slides, since the serum proteins present seemed to render the surfaces essentially the same⁴. Lacuna formation was also observed in blood contained in a capillary tube. It took longer for the lacunae to appear after incubation at room temperature than at 37.5° C. At 5° C lacuna formation was inhibited, in keeping with the known reduction in leukocyte adherence⁵ at this temperature. Both heparinised and EDTA-treated blood were used and the red cells could be suspended successfully in a variety of diluents including Tyrode's and colloidal solutions. Using 1% phosphate buffered saline solutions it was found that acidic preparations were unsatisfactory but those at or above pH 7 were satisfactory. Use of borate buffers at pH 9 and 10 favoured early formation of lacunae. Red cell dilution was optimal between 1/6 and 1/32, the critical determinant being the achievement of a monocell layer of erythrocytes.

Individual differences in lacuna formation were noted, some of the most rapidly formed lacunae being found in patients with acute infectious disease. The use of vital stains to differentiate the cells did not interfere with lacuna production (Fig. 2a, b).

It was possible to induce streaming of the ambient red cells by applying gentle pressure on the coverslip. Although this resulted in an elliptical distortion of the lacuna it did not dislodge the central leukocyte, indicating that it is adherent. The significance of this adherence in the formation of lacunae was demonstrated in thick preparations, in which free floating non-adherent leukocytes could be seen unassociated with lacuna formation (Fig. 2a), at the same time that deeper focus on the same field (Fig. 2b) disclosed a single adherent leukocyte within each lacuna.

The mechanism of lacuna formation remains obscure. Although it can be argued that the weight of the coverslip⁶ compresses the leukocyte to balloon dimensions, or that during adherence its hyaloplasm⁷ extends to fill the entire lacuna, the cell body as seen with thiazine red R supravital staining, as well as with phase, is significantly smaller than the lacunar space. At first glance, these lacunae also resemble plaques of immune haemolysis⁸ but the lacunae we observed are much smaller and phase microscopy reveals

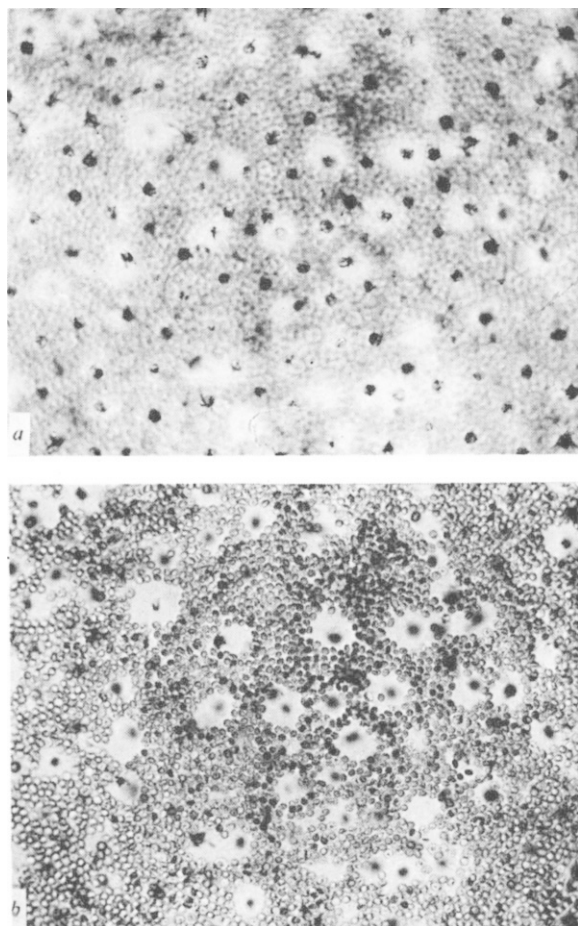


Fig. 2 Lacuna formation with leukocytes stained supravitaly with idonitrotetrazolium. Exactly the same field is seen at two levels. *a*, At higher level non-adherent floating leukocytes are seen with no relation to lacunar spaces. Lacunae are not in focus. $\times 174$. *b*, At deeper level lacunae with central adherent leukocytes can be seen.

no haemolytic ghost cells within them⁹. Further, no foreign mixture of cells was used. Electrostatic forces could be deemed responsible for the erythrocyte repulsion, but changing the charge on the red cell by the addition of formalin or salmine sulphate (10^{-4}) to the diluent had no effect.

It seems more likely to me that the adherent leukocyte secretes a substance which produces a territorial space about it. In support of this is the fact that lacuna formation is accentuated by suspending erythrocytes in a borate buffer at pH 10 which could be expected to produce cell irritation. Rosenberg¹⁰ has demonstrated by ellipsometry that the leukocyte can secrete a micro-exudate when in contact with glass, and this material can spread out to several cell diameters. Taylor later visualised such a cell exudate by immunological means¹¹ and observed that, by virtue of such liberated products, human conjunctival cells inhibit the spreading and contact relations of themselves and nearby cells¹².

In the monocellular layering technique described here, precisely such a leukocyte exudate may be responsible for the lacunae, the absence of erythrocytes indicating a secretion which inhibits their regular distribution and movement. I have been uniformly unsuccessful in staining or visualising any such material in the lacuna. Nonetheless, fixed slides, subsequently washed, show no cells but show clear lacunae by negative contrast when Giemsa stain is used and the slide viewed under phase. Further supporting the view that a secretion could be responsible is the fact that slides with lacunae, washed and resurfaced with red cells, show reformation of lacunae. Finally, it should be pointed out

that lacuna formation was not dependent on the presence of erythrocytes, but rather on the adherent leukocyte. Thus, lacunae could be seen when either carbon particles (India ink 1/12), polystyrene latex particles (1.011 μ m diameter, 10%, Dow), or colloidal silica (Ludox S 1/30, Dupont) was used in place of erythrocytes as a field indicator.

Further study of lacuna formation seems warranted since these lacunae may provide another source of information of the behaviour of the leukocyte in disease states and in the immune response.

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- ¹ Bryant, R. E., Des Prez, R. M., Van Way, M. H., and Rogers, D. E., *J. exp. Med.*, **124**, 483 (1966).
- ² Shelley, W. B., *Am. J. clin. Path.*, **39**, 433 (1963).
- ³ Bessis, M., *Cytology of the Blood and Blood-Forming Organs* (Grune and Stratton, New York, 1956).
- ⁴ Manley, R. S., *Adhesion in Biological Systems* (Academic Press, New York, 1970).
- ⁵ Bryant, R. E., and Sutcliffe, M. C., *Proc. Soc. Biol. Med.*, **141**, 196 (1972).
- ⁶ Rabinowitz, Y., and Schrek, R., *Blood*, **20**, 453 (1962).
- ⁷ Bessis, M., in *The Cell* (edit. by Brachet, J., and Mirsky, A. E.), **5**, 163 (Academic Press, New York, 1961).
- ⁸ Jerne, N. K., and Nordin, A. A., *Science, N.Y.*, **140**, 405 (1963).
- ⁹ Ingraham, J. S., and Bussard, A., *J. exp. Med.*, **119**, 667 (1964).
- ¹⁰ Rosenberg, M. D., *Biophysical J.*, **1**, 137 (1960).
- ¹¹ Taylor, A. C., *Expl. Cell Res.*, Suppl. 8, 154 (1961).
- ¹² Taylor, A. C., in *Biological Interactions in Normal and Neoplastic Growth* (edit. by Brennan, N. J., and Simpson, W. L.), 169 (Little, Brown, and Co., Boston, 1962).

Is the Area Postrema a Control Centre of Blood Pressure?

SEVERAL antihypertensive drugs have a central site of action¹. The anatomical structures involved are unknown but there is evidence that some areas in the vicinity of the fourth brain ventricle mediate their effects¹. Also in the region of the fourth ventricle noradrenaline²⁻⁶ and γ -aminobutyric acid⁷ bring about a depression of the cardiovascular system.

Recently it has been reported that the area postrema, which lies outside the blood-brain barrier⁸ and protrudes into the fourth ventricle, mediates the central cardiovascular response to angiotensin II in the dog⁹. The area postrema bears a close histological resemblance to the carotid body¹⁰ and has been shown to act as a chemoreceptor¹¹. We have destroyed the area postrema of the rat and report here the effect of this operation on the blood pressure.

Male Sprague-Dawley normotensive rats and spontaneously hypertensive (SH) rats of the strain developed by Okamoto and Aoki¹² were used. The destruction of the area postrema was performed by sight through the occipital foramen by thermocoagulation. The procedure has been described in detail previously¹³. With the exception of thermocoagulation, the sham operation was exactly similar to the true operation. The blood pressure in unanaesthetised rats was measured indirectly by a tail cuff method¹³ and in anaesthetised animals (pentobarbital, 40 mg kg⁻¹, i.p.) directly from the carotid artery.

In 1 week the ablation of the area postrema induced an elevation of blood pressure both in initially normotensive (Fig. 1) and in SH rats (Fig. 2). Five weeks after the operation the blood pressure of animals with the area postrema destroyed was still greater than that of the controls. Although the rats were accustomed to the blood-pressure measurements before the operation, most of them

were somewhat excited at the start of each measurement, that is for the first 2 to 3 min. At 1 week the first recordings in normotensive controls were 7 ± 2 mm Hg ($n=15$) higher

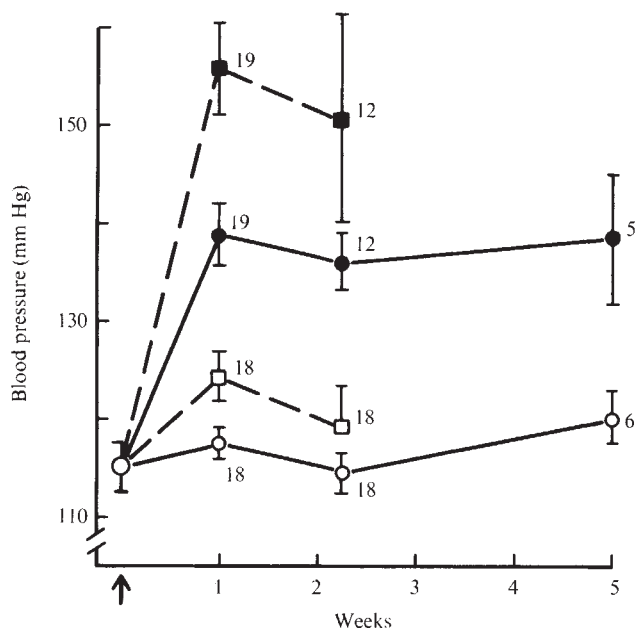


Fig. 1 Influence of the ablation of area postrema (■, ●) and sham operation (□, ○) on the blood pressure of normotensive rats. ---, Values recorded during the initial 2 to 3 min period of the measurement procedure; —, the following, stabilised values. Vertical bar, s.e.m. The number of rats is given on the figure.

than the stabilised values 3 to 4 min later (Fig. 1), whereas in the area postrema ablated rats, the initial values were 17 ± 3 mm Hg ($n=18$) higher than the stabilised ones. In SH rats, the corresponding differences were 9 ± 4 mm Hg ($n=14$) and 30 ± 5 mm Hg ($n=19$), (Fig. 2). In sham-operated rats the greatest difference between the first recordings and the stabilised value was 15 mm Hg, but in rats with the area postrema destroyed differences up to 60 mm Hg were measured. The initial pressure recordings remained higher than the later ones for at least 2 weeks. During the first measurement procedures, some of the operated rats died of pulmonary oedema. Shortly before death in SH rats, values up to 270 mm Hg, and in initially normotensive rats up to 200 mm Hg, were measured. No such blood pressure elevations or sudden deaths occurred in the sham-operated rats.

About 3 weeks after the destruction of the area postrema the initially normotensive rats also showed an elevated blood pressure during anaesthesia (sham operated, 126 ± 8 mm Hg ($n=9$); area postrema ablated, 147 ± 6 mm Hg ($n=11$), $P < 0.05$).

The area postrema may exert a tonic inhibition on the blood pressure in the rat as its ablation induced an elevation of the blood pressure lasting at least 5 weeks. The area postrema might also be involved in the feed-back control of blood pressure as, in the absence of this region, very high blood pressures were induced by a stress situation, that is, by the recording of blood pressure.

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- Van Zwieten, P. A., *J. Pharm. pharmac.*, **25**, 89 (1973).
- McCubbin, J. W., Kaneko, Y., and Page, I. H., *Circ. Res.*, **8**, 849 (1960).
- Kaneko, Y., McCubbin, J. W., and Page, I. H., *Circ. Res.*, **8**, 1228 (1960).
- Nashold, B. S., Mannarino, E., and Wunderlich, H., *Nature*, **193**, 1297 (1962).
- Share, N. N., and Melville, K. I., *J. Pharmac. exp. Ther.*, **141**, 15 (1963).
- Smookler, H. H., Severs, W. B., Kinhard, W. J., and Buckley, J. P., *J. Pharmac. exp. Ther.*, **153**, 485 (1966).
- Bhargava, K. P., Bhattacharya, S. S., and Srimal, R. C., *Br. J. Pharmac.*, **23**, 383 (1964).
- Wislocki, G. B., and Putnam, T. J., *Anat. Rec.*, **19**, 281 (1920).
- Joy, M. D., and Lowe, R. D., *Nature*, **228**, 1303 (1970).
- De Kock, L. L., *Acta anat.*, **37**, 265 (1959).
- Borison, H. L., and Brizzee, K. R., *Proc. Soc. exp. Biol. Med.*, **77**, 38 (1951).
- Okamoto, K., and Aoki, K., *Jap. Circ. J.*, **27**, 282 (1963).
- Ylitalo, P., *Ann. Med. exp. Fenn.*, **50**, 195 (1972).
- Friedman, M., and Freed, S. C., *Proc. Soc. exp. Biol. Med.*, **70**, 670 (1949).

Mechanism of the Viscoelastic Deformation of Collagenous Tissue

THE purpose of the experiment reported here was to examine the striking observation of Rigby *et al.*¹ that for collagenous tissue the thermally activated process controlling mechanical relaxation changes abruptly at the body temperature. For rat tail tendon it was observed¹ that below 37° C the process is entropy activated: the activation energy $\Delta H = 0$. Above 37° C, Rigby *et al.*¹ conclude that ΔH is large although their

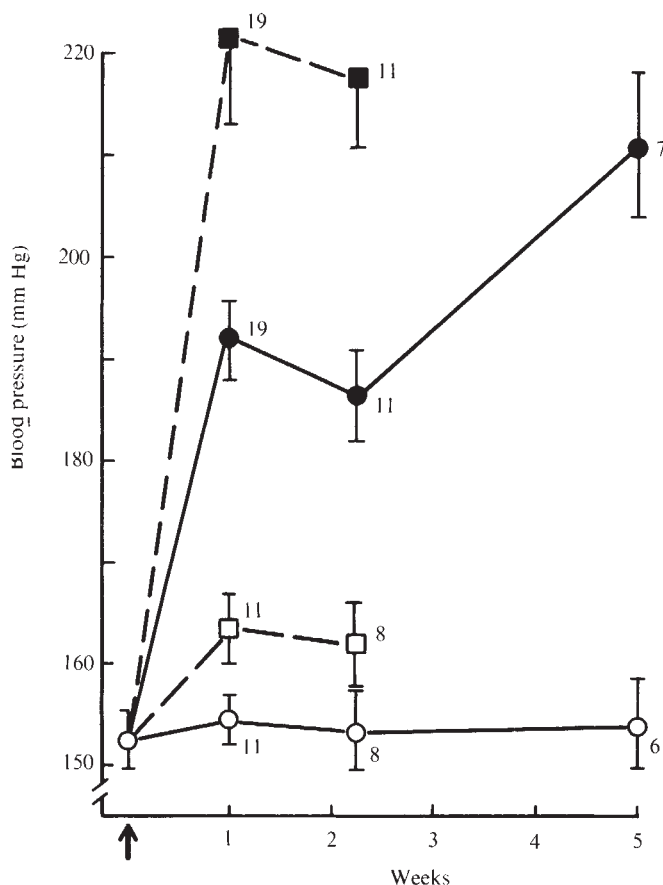


Fig. 2 Influence of the ablation of area postrema (■, ●) and sham operation (□, ○) on the blood pressure of spontaneously hypertensive rats. Symbols as in Fig. 1.

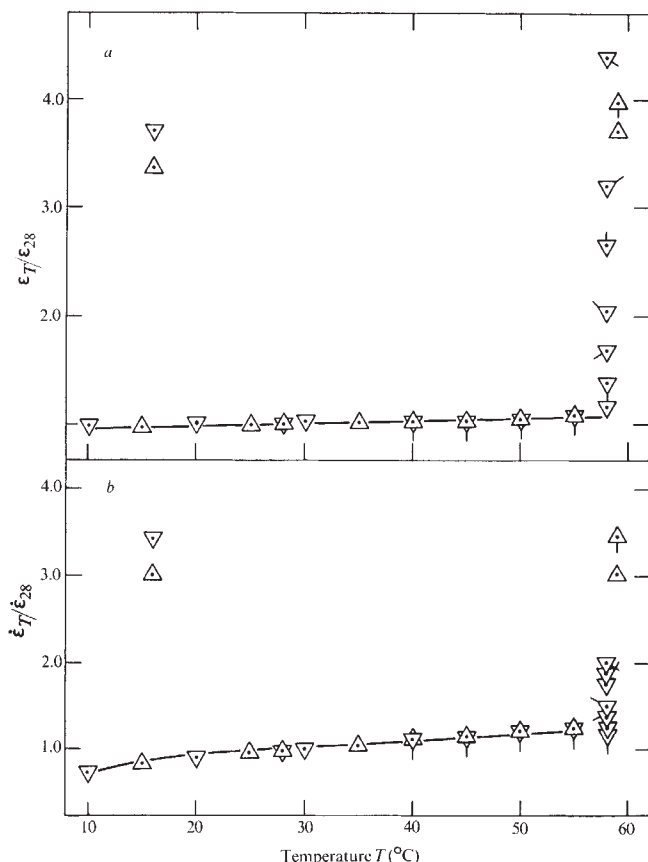


Fig. 1 Temperature dependence of normalised isochronal strain (a) and isochronal strain rate (b) at $t=20$ s for two specimens of digital tendon. Repeated experiments at a given temperature are indicated by the pips on the triangles, beginning with pip downwards for the second experiment and progressing clockwise by 60° for each successive experiment. Tendons studied under constant loads of 0.72 kg (male, 55 yr) and 0.30 kg (female, 89 yr). a: ∇ , Male, $\epsilon_{28}=1.40\%$; $\triangle$, female, $\epsilon_{28}=1.42\%$. b: ∇ , Male, $\dot{\epsilon}_{28}=5.81 \times 10^{-3} \% s^{-1}$; $\triangle$, female, $\dot{\epsilon}_{28}=4.00 \times 10^{-3} \% s^{-1}$.

experiment does not yield a quantitative result. The result is paradoxical in two ways. First, entropy activation, although feasible conceptually, is essentially unknown in nature. Second, is it plausible that the rate process should change in such a profound way right at the body temperature? We have now resolved both paradoxes.

The measurements were made on human flexor digitorum sublimus tendon *in vitro*. The specimen was mounted in a creep apparatus in which it could be subjected to a constant tensile load, and the tensile strain measured as a function of time of application of load, t . The specimen was immersed in 0.9% saline solution buffered to pH 7 which was maintained at a constant temperature in the range $10^\circ C$ to $60^\circ C$. Figure 1 shows data for two specimens, one from a male of age 55 yr and the other a female of age 89 yr. The strain at $t=20$ s observed at T , ϵ_T , is divided by the strain at $t=20$ s observed at $28^\circ C$, ϵ_{28} , and plotted against T in Fig. 1a. It will be seen that (1) there is no discontinuity in the region of $37^\circ C$; (2) the data for both specimens are in excellent agreement; and (3) there is a transition at $58^\circ C$. The temperature dependence of the creep rate $\dot{\epsilon}_T$ supports these conclusions. This is illustrated in Fig. 1b in which $\dot{\epsilon}_T/\dot{\epsilon}_{28}$ is plotted against T .

It may be concluded that for digital tendon there is no clear evidence for a change in rate process at $37^\circ C$; however, in order to test further the conclusion of Rigby *et al.*¹ that $\Delta H=0$ below $37^\circ C$ we measured ΔH at $28^\circ C$ by observing the change in creep rate induced by a small abrupt change of temperature (T -jump experiment). It follows from the theory of reaction rates^{2,3} that if during a creep experiment the tempera-

ture is changed abruptly from T_0 to T at time t' and if $\dot{\epsilon}_{T_0}$ and $\dot{\epsilon}_T$ are the measured strain rates at T_0 and T at time t' , then

$$\dot{\epsilon}_T/\dot{\epsilon}_{T_0} = \exp[(\Delta H/R)(1/T_0 - 1/T)] \quad (1)$$

Hence,

$$\ln(\dot{\epsilon}_T/\dot{\epsilon}_{T_0}) = (\Delta T/RT T_0) \Delta H \quad (2)$$

in which $\Delta T = (T - T_0)$ and R is the gas constant. In our experiment a number of T -jumps were performed from a fixed $T_0 = 28^\circ C$. According to equation (2) a plot of $\ln(\dot{\epsilon}_T/\dot{\epsilon}_{T_0})$ against $\Delta T/RT T_0$ should be linear and of slope ΔH . In each experiment the temperature was changed at a time $t'=60$ s after application of the stress. Fig. 2 shows results for the two specimens already described and a specimen taken from a male of 21 yr. It will be seen that the data for all three specimens are in good agreement and yield a finite value of ΔH , 12 kcalorie mol^{-1} at $T_0=28^\circ C$.

It follows from this result that for human digital tendon the conclusion of Rigby *et al.*¹ does not hold. The discrepancy between the two experiments is difficult to explain. It is certainly not due to the use of different mechanical techniques, stress relaxation¹ as opposed to creep. It is quite possible that since ΔH is low, and since Rigby *et al.*¹ studied many specimens (rat tail tendon breaks easily in saline solution and for this reason was not used in our experiments), the random scatter of results obscured the small temperature dependence of the stress relaxation modulus. This would explain the conclusions of Rigby *et al.* below $37^\circ C$. The cause of the sudden increase in the temperature dependence of stress relaxation modulus above $37^\circ C$ observed by Rigby *et al.*¹ in rat tail tendon is unexplained. It could be due to the age of the tendon, since their specimens were taken from animals of age approximately 3 months.

A complete description of our work will be presented elsewhere including a study of the shrinkage transition at $58^\circ C$ using the T -jump technique. This transition is rate activated with $\Delta H \sim 250$ kcalorie mol^{-1} . It is our belief that for pseudo-first order transitions of this type and also for mechanical relaxation the T -jump technique is of considerable potential

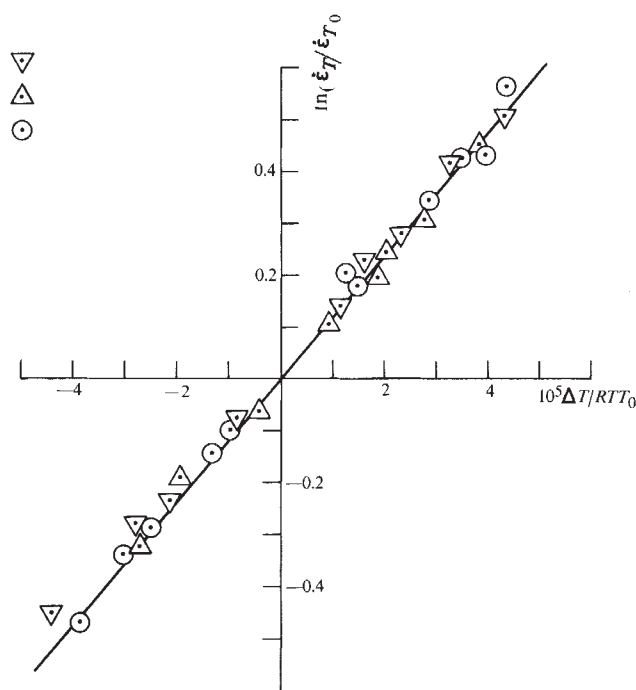


Fig. 2 Dependence of $\ln \dot{\epsilon}_T/\dot{\epsilon}_{T_0}$ for three specimens of digital tendon on $\Delta T/RT T_0$. Straight line drawn according to equation (2) with $\Delta H=12$ kcalorie mol^{-1} . Tendons studied under constant loads of 0.72 kg (male, 55 yr (∇)), 0.30 kg (female, 89 yr ($\triangle$)) and 0.76 kg (male, 21 yr ($\circ$)). $T_0=28^\circ C$.

for the study of biological systems. The linear viscoelastic techniques such as time-temperature superposition, which have found wide application for determining ΔH in synthetic polymers^{4,5}, are not applicable to biological systems. The strength of the T -jump technique is that even when the structure of the specimen changes during the deformation process (as it does in tendon⁶) the activation energy may still be determined since the creep rates $\dot{\epsilon}_{T_0}$ and $\dot{\epsilon}_T$ are determined at the same structure: that which occurs at the time of the T -jump. The technique is also accurate for low values of ΔH when superposition (as in the experiments of Rigby *et al.*¹) becomes inaccurate.

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- ¹ Rigby, B. J., Hirai, R., Spikes, J. D., and Eyring, H., *J. gen. Physiol.*, **43**, 265 (1959).
- ² Glasstone, S., Laidler, K. J., and Eyring, H., *The Theory of Rate Processes* (McGraw-Hill, New York, 1941).
- ³ Tietz, T. E., and Dorn, J. E., *Trans. Am. Inst. Min. metall. Engrs.*, **206**, 156 (1956).
- ⁴ Ferry, J. D., *Viscoelastic Properties of Polymers*, second ed. (Wiley, New York, 1970).
- ⁵ McCrum, N. G., Read, B. E., and Williams, G., *Anelastic and Dielectric Effects in Polymeric Solids* (Wiley, London, 1967).
- ⁶ Diamant, J., Keller, A., Baer, E., Litt, M., and Arridge, R. G. C., *Proc. R. Soc.*, **B180**, 293 (1972).

Ion Beam Etching of Red Blood Cells and Latex Spheres

ION etching techniques have recently been utilised in attempts to reveal subsurface cell structures in the scanning electron microscope¹⁻⁷. There has, however, been some difficulty in distinguishing intracellular structures from artefacts produced by the ion etching process. This inability to distinguish cellular structures from features produced by the ion beam suggests that a better understanding of the ion etching process as it is applied to organic material is required before this technique can be applied to the recognition and investigation of biological cell types and structure. In our study we examined the etch effects produced in normal red blood cells and homogeneous latex spheres by a variety of ion beam etching conditions.

Initial glow discharge ion etching studies of red blood cells showed that the etched red cell consisted of cone-like or filament-like structures¹. Later studies^{2,5-7} demonstrated that

these filament-like structures and similar ridge-like features found in other etched cells²⁻⁶ and tissues⁷ were produced by the ion etching process². The presence of these cone-like structures in ion etched cells and tissue has hindered the identification of cellular components. The study reported here was carried out to determine the ion etching conditions which reduce cone formation in red blood cells and homogeneous latex spheres.

The red cell fraction of normal whole blood was washed in an isotonic phosphate-buffered medium (10 mM KCl, 141 mM NaCl, 1 mM MgCl₂, 1.3 mM CaCl₂, 0.8 mM NaH₂PO₄, 5 mM Na₂HPO₄ at pH 7.4, 300 mOsm) and fixed for 30 to 60 min in 0.5% phosphate-buffered glutaraldehyde, pH 7.4, 320 mOsm. The cells were then washed in deionised water and allowed to settle from a droplet of water onto a silicon disk. Care was taken that air drying did not occur during this time or during subsequent procedures. Dehydration was carried out by bathing the cells in 20, 50, 70, 90 and 100% ethanol solutions and 50 and 100% amyl acetate solutions for 5 min periods. The cells were taken directly from the 100% amyl acetate solution and critical point dried in carbon dioxide.

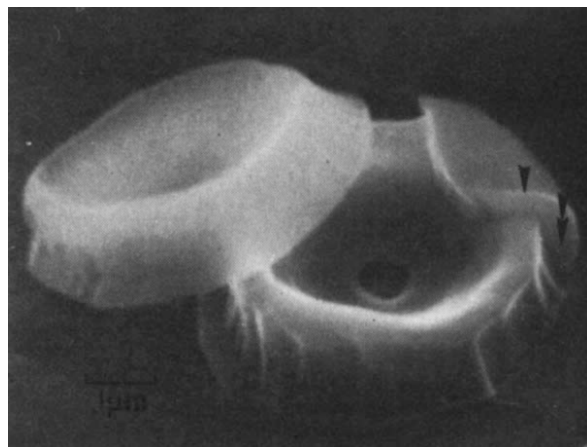


Fig. 2 Red cell ion beam etched by 17 kV argon ion beam with 90° angle of incidence for 3 min. A portion of the bottom cell which was shielded from the ion beam is unetched.

Styrene divinylbenzene copolymer latex spheres, 4 to 8 μ m in diameter, were obtained from the Dow Chemical Company. The latex spheres were dispersed in deionised water, deposited on a silicon disk and air dried.

Ion beam etching was carried out in the specimen chamber of an ETEC Autoscan scanning electron microscope using an ETEC ion beam etching device with argon gas. The ion beam had an estimated diameter of 2 mm and a 2.3° beam divergence. The specimen was positioned about 1 inch from the ion gun aperture. Ion accelerating voltages of 5 to 20 kV with ion currents of 0.5 to 20 μ A, respectively, were used for etching. The angle of incidence was maintained at 90° and etching times ranged from 1 to 30 min.

After etching, the red cells and latex spheres were coated with thin films of carbon and gold or gold-palladium and examined in the scanning electron microscope at 20 kV.

Red blood cells etched by a 6 kV argon ion beam had a very irregular surface characterised by cone- or ridge-like structures (Fig. 1) similar to those seen in cells etched by 1 to 4 kV argon ion beams^{2,6}. There has been some indication in previous studies that the texture of the etched cell surface becomes smoother as the ion beam accelerating voltage increases². The change in the texture of the etched cell surface with increasing ion accelerating voltage can be seen by comparing the cell in Fig. 1 with the cells in Fig. 2 which were etched by a 17 kV argon ion beam. Although the cells in Fig. 2 lacked much of

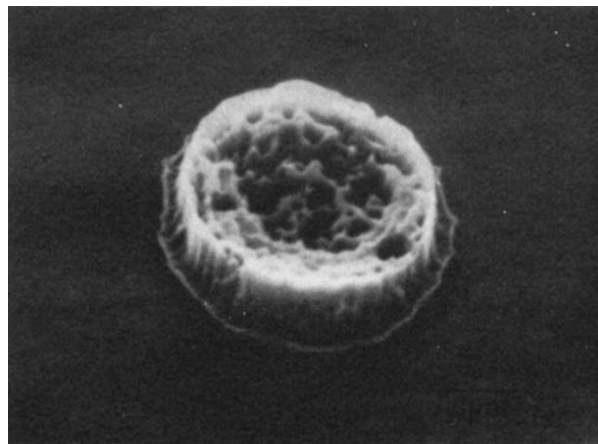


Fig. 1 Normal red blood cell ion beam etched by a 6 kV argon ion beam at 90° angle of incidence for about 25 min.

the surface structure present in the cells in Fig. 1, the cells in both figures had similar groove-like features along their perimeter. These grooves were always found to be oriented in the direction of the ion beam.

The configuration of the cells in Fig. 2 during etching was such that a portion of the bottom cell was shielded from the ion beam. It is possible to see a cross-section of the portion of the cell that remained unetched. The shape of the etched sections of the cells suggests that different amounts of material were removed from different areas of the cells. It seems that less material has been removed from areas where the ion beam intersected the cell surface at 90° (indicated by single arrow) than from areas where the ion beam had about a 45° angle of incidence with the surface of the cell (double arrow).

The variation in the sputtering yield (number of atoms removed for each incident ion) with angle of incidence suggested in Fig. 2 is similar to the results of earlier studies of ion beam etching of metals⁸. The shapes of etched metal spheres inserted into ion beams demonstrated that the sputtering yield was a minimum at 90°, increased to a maximum at from 20° to 40°, and decreased again at grazing incidence.

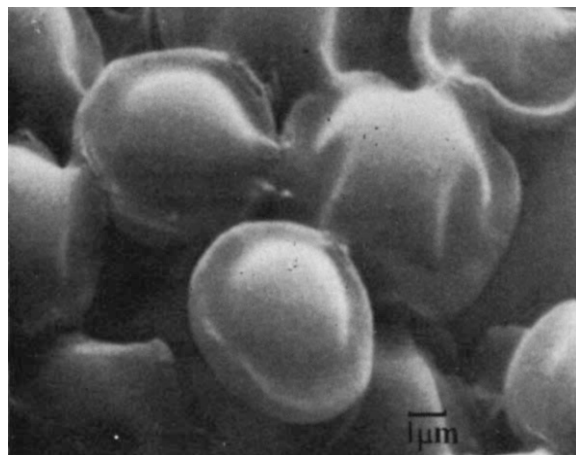


Fig. 3 Latex spheres etched by a 17 kV argon ion beam at 90° angle of incidence.

The effects of the change in sputtering produced by a change in angle of incidence along a curvilinear surface can also be seen in etched latex spheres (Fig. 3). Ion beam etched latex spheres are comparable to metal spheres which were ion beam etched in earlier studies⁸. The surfaces of the etched latex spheres had a smooth texture similar to that of red cells etched under similar conditions (Fig. 2) and occasionally had groove-like features somewhat similar to the features along the perimeter of the etched red cells.

The results of this study demonstrate that cone- and ridge-like structures present in red cells etched by relatively low voltage (1 kV to 6 kV) ion beams are reduced as the ion accelerating voltage is increased (for example, to 17 kV). The results further demonstrate that the shapes of red blood cells and homogeneous latex spheres etched with ion beams are comparable and can be explained by the change in the sputtering yield with the variation of the angle of incidence along the red cell and latex sphere surface. This relationship between sputtering yield and angle of incidence is similar to that determined earlier for inorganic materials. The results of the red cell etching are consistent with the view that the normal red cell is homogeneous, having no distinct subsurface structure resolvable in the scanning electron microscope. Work now in progress is using the ion beam etching technique to study the structure of nucleated and parasitised red cells.

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- ¹ Lewis, S. M., Osborn, J. S., and Stuart, P. R., *Nature*, **220**, 614 (1968).
- ² Baker, R. F., in *Red Cell Membrane* (edit. by Jamieson, G. A., and Greenwalt, T. J.), 13 (Lippincott, Philadelphia, 1969).
- ³ Ambrose, E. J., Batzdorf, U., Osborn, J. S., and Stuart, P. R., *Nature*, **227**, 397 (1970).
- ⁴ Holt, P. J. L., Pal, S. G., Catovsky, D., and Lewis, S. M., *Clin. exp. Immun.*, **10**, 555 (1972).
- ⁵ Hodges, G. M., Muir, M. D., Sella, C., and Carteau, A. J. P., *J. Microsc.*, **95**, 445 (1972).
- ⁶ Fulker, M. J., and Holland, L., *Micron*, **2**, 117 (1971).
- ⁷ Fulker, M. J., Holland, L., and Hurley, R. E., in *Scanning Electron Microscopy, 1973* (edit. by Johari, O., and Corvin, I.), 380 (IIT Research Institute, Chicago, 1973).
- ⁸ Wehner, G. K., *J. appl. Phys.*, **30**, 1762 (1959).

Optically Detected Conformational Changes in Haemoglobin Single Crystals

THERE is considerable X-ray evidence that the haem stereochemistry is different in oxy- and deoxyhaemoglobin¹⁻³. This structural change is an important part of the oxygenation process, because it is believed to induce conformational changes in the surrounding protein that are responsible for cooperativity. We have investigated conformational changes in the haem region by measuring polarised absorption spectra on single crystals. Using this technique we have detected changes in porphyrin plane orientation associated with an alteration of the ligand, spin, and oxidation state of the haem iron, factors which influence haem stereochemistry. Our results not only show for the first time that contacts between the porphyrin and amino acid side chains change upon oxygenation, but they are also consistent with the Hoard-Perutz hypothesis on the nature of the stereochemical trigger¹⁻³.

Crystals of horse oxyhaemoglobin, carbonmonoxyhaemoglobin, and methaemoglobin were prepared according to Perutz⁴, except that the oxy and carbonmonoxy forms were crystallised at 4° C in order to minimise oxidation to methaemoglobin. Crystals of cyanide, azide, fluoride, and formate complexes were formed by diffusing the ligand into the methaemoglobin crystal. The deoxy form was prepared by dithionite reduction of methaemoglobin crystals previously modified with bis(N-maleimidomethyl)ether (BME)⁵. Single crystal spectra were measured over a wide wavelength range (230–1,200 nm) with plane-polarised light incident normal to the (001) face of the horse haemoglobin crystal⁴ of space group C2. The measurements were made with a microspectrophotometer similar to the one previously described⁶.

The important experimental parameter of the single crystal spectrum is the polarisation ratio. It is defined at each wavelength as the ratio of the optical density measured with the electric vector of the plane-polarised light aligned parallel to the *b* crystal axis to the optical density measured with the electric vector parallel to the *a* crystal axis. The polarisation ratio in

the purely haem absorption region depends only on geometric factors. These are the molecular directions of the haem complex in which plane-polarised light is maximally absorbed and the orientation of these directions with respect to the *a* and *b* crystal axes. For the intense Soret band near 400 nm these molecular directions are very accurately described in all derivatives by a plane parallel to the mean plane of the 24 porphyrin ring atoms. This means that no light is absorbed for the component of the electric vector perpendicular to the porphyrin plane, while all directions contained in this plane show equal absorption. This planar-absorber behaviour for the Soret band has been established from the excellent agreement of the optically-determined porphyrin plane orientation⁷⁻⁹ (Makinen and Eaton, unpublished) with that found by X-ray diffraction¹⁰⁻¹⁴ and electron paramagnetic resonance¹⁵ studies on a variety of haem protein crystals. Thus, we interpret differences in Soret polarisation ratio among haemoglobin derivatives as arising from differences in porphyrin plane orientation.

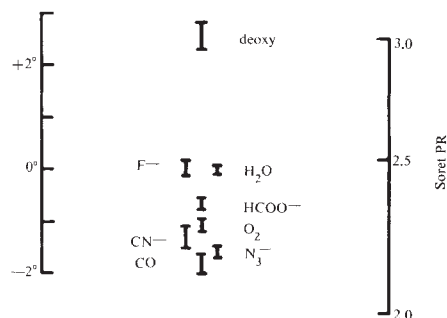


Fig. 1 Soret polarisation ratio and relative porphyrin plane orientation in horse haemoglobin derivatives. The uncertainties represent the average deviation from the mean of measurements on at least two crystals. The minimum angle between porphyrin plane normals, ϕ , is calculated from equation (1) and is relative to the porphyrin normal of (aquo) methaemoglobin. The following abbreviations are used: deoxy (deoxyBMEhaemoglobin), H_2O (aquo) methaemoglobin, O_2 (oxyhaemoglobin), CO (carbonmonoxyhaemoglobin), CN^- (cyanomethaemoglobin), F^- (fluoridomethaemoglobin), HCOO^- (formatomethaemoglobin), N_3 (azidomethaemoglobin).

Our results are summarised in Fig. 1. The scale at the right is the average polarisation ratio for the Soret band. The scale at the left shows the minimum change in orientation of the porphyrin plane that can account for the observed change in polarisation ratio. The angles are referred to the porphyrin normal of methaemoglobin and are calculated from the relation

$$\phi_{ij} = \arctan(\text{PR}_i) - \arctan(\text{PR}_j) \quad (1)$$

where $(\text{PR})_i$ and $(\text{PR})_j$ are the Soret polarisation ratios for the *i*th and *j*th derivatives. In deriving this expression we have assumed D_2 (222) symmetry for the orientation of the four porphyrin planes in all derivatives. This symmetry is exact for methaemoglobin within the experimental errors of both the electron paramagnetic resonance results¹⁵ and the 2.8 Å atomic coordinates provided to us by M. F. Perutz. It is important to note that any deviation from D_2 symmetry for either the *i*th or *j*th derivatives will result in an increased absolute value of ϕ_{ij} for either α or β haems, or both. If we assume, for example, that only α or only β haems change orientation, then the absolute values of ϕ_{ij} are doubled. Also, changes in porphyrin plane orientation can be assumed to take place relative to axes fixed within the subunits since X-ray data^{4,16,18} show that the quaternary structures of methaemoglobin, azidomethaemoglobin, oxyhaemoglobin, carbonmonoxyhaemoglobin, and deoxyBMEhaemoglobin are essentially identical.

Our interpretation of the variation in Soret polarisation ratio among haemoglobin derivatives receives strong support from X-ray results. The minimum angle between the porphyrin normals of methaemoglobin and deoxyhaemoglobin is cal-

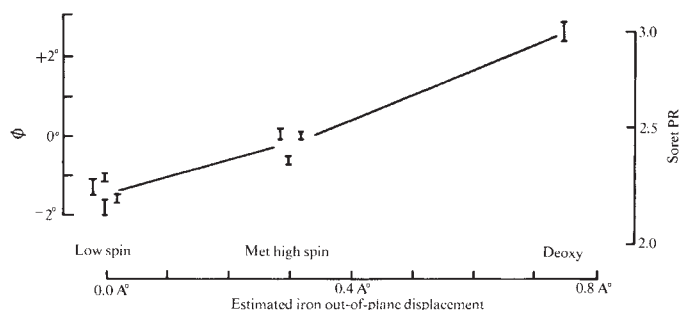


Fig. 2 Soret polarisation ratio and relative porphyrin plane orientation against estimated displacement of the iron from the mean porphyrin plane. The displacement in horse methaemoglobin and deoxyhaemoglobin is 0.3 Å and 0.75 Å, respectively². To assign approximate values for the other derivatives we have appealed to the X-ray data on sea lamprey cyanomethaemoglobin¹⁹ and the predictions from small molecule structures². Thus, we use 0.75 Å, 0.3 Å, and 0.0 Å for ferrous high spin (deoxy), ferric high spin (H_2O , F^- , HCOO^-), and ferric or ferrous low spin derivatives (O_2 , CO , CN^- , N_3), respectively. The polarisation ratios (PR) are shown as clustered around these points for clarity.

culated to be $+2.5^\circ$ (Fig. 1). This value is consistent in both sign and magnitude with the movement of the porphyrin planes detected by difference Fourier studies^{2,16} (M. F. Perutz, personal communication). The only other haemoglobin derivative to be examined in the liganded quaternary structure at high resolution by X-ray methods is horse carbonmonoxyhaemoglobin. Here again the optical and X-ray results are in agreement (E. G. Heidner, personal communication). Oxyhaemoglobin has not yet been studied at high resolution because the crystals are almost completely oxidised to methaemoglobin by the end of the X-ray exposure². The much smaller crystals used in our optical experiments contained less than 20% methaemoglobin. We find that the porphyrin planes tilt at least 3.5° in the transition from oxyhaemoglobin to deoxyhaemoglobin constrained by BME to remain in the liganded quaternary structure. This rotation corresponds to a displacement of peripheral porphyrin atoms of at least 0.3 Å. Since there are a large number of non-bonded interactions between the protein and porphyrin—there are approximately sixty atoms of the protein less than 4.0 Å from the porphyrin²⁰—such a displacement must be accompanied by a movement of amino acid side chains in contact with the porphyrin ring. Thus, our observation of a change in orientation of the porphyrin plane supplies the first experimental evidence for a change in porphyrin-protein contacts upon oxygenation.

Hoard^{1,3} and Perutz^{2,16} have suggested that the important structural change initiating cooperativity in the protein is a shortening of the distance between the haem-linked histidine and the porphyrin plane upon oxygen binding. The histidine is covalently bonded to the iron, so that the principal part of this movement arises from the shift of the iron from 0.75 Å out of the plane in deoxyhaemoglobin to an essentially coplanar position in oxyhaemoglobin. This histidine-porphyrin movement, referred to as the stereochemical trigger, is expected to force changes in the contacts between the porphyrin and amino acid side chains¹⁶. From this stereochemical trigger hypothesis we would predict that derivatives with similar iron out-of-plane displacements should have similar porphyrin orientations. As a test of this prediction we have plotted our orientation results against the estimated iron out-of-plane displacement (Fig. 2). The data do indeed neatly divide into three classes according to the iron-porphyrin distance. This result is not only consistent with the postulated importance of the histidine-porphyrin movement, but it also suggests that this stereochemical change is transmitted to the protein through an alteration in porphyrin-protein contacts.

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- ¹ Hoard, J. L., in *Hemes and Hemoproteins* (edit. by Chance, B., Estabrook, R. W., and Yonetani, T.), 9 (Academic Press, New York, 1966).
- ² Perutz, M. F., *Nature*, **228**, 726 (1970).
- ³ Hoard, J. L., *Science N.Y.*, **174**, 1295 (1971).
- ⁴ Perutz, M. F., *J. Cryst. Growth*, **2**, 54 (1968).
- ⁵ Simon, S. R., and Konigsberg, W. H., *Proc. natn. Acad. Sci. U.S.A.*, **56**, 749 (1966).
- ⁶ Eaton, W. A., and Lewis, T. P., *J. chem. Phys.*, **53**, 2164 (1970).
- ⁷ Eaton, W. A., and Hochstrasser, R. M., *J. chem. Phys.*, **46**, 2533 (1967).
- ⁸ Eaton, W. A., and Hochstrasser, R. M., *J. chem. Phys.*, **49**, 985 (1968).
- ⁹ Makinen, M. W., and Eaton, W. A., *Ann. N.Y. Acad. Sci.* (in the press).
- ¹⁰ Stryer, L., Kendrew, J. C., and Watson, H. C., *J. molec. Biol.*, **8**, 96 (1964).
- ¹¹ Nobbs, C. L., Watson, H. C., and Kendrew, J. C., *Nature*, **209**, 339 (1966).
- ¹² Bretcher, P. A., thesis, Cambridge Univ., 1968.
- ¹³ Watson, H. C., *Prog. Stereochem.*, **4**, 298 (1970).
- ¹⁴ Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E., *J. biol. Chem.*, **246**, 1511 (1971).
- ¹⁵ Bennett, J. E., Gibson, J. F., and Ingram, D. J. E., *Proc. R. Soc.*, **A240**, 67 (1957).
- ¹⁶ Perutz, M. F., and TenEyck, L. F., *Cold Spring Harbor Symp. quant. Biol.*, **36**, 569 (1971).
- ¹⁷ Simon, S. R., Konigsberg, W. H., Bolton, W., and Perutz, M. F., *J. molec. Biol.*, **28**, 451 (1967).
- ¹⁸ Perutz, M. F., and Mathews, F. S., *J. molec. Biol.*, **21**, 199 (1966).
- ¹⁹ Hendrickson, W. A., Love, W. E., and Karle, J., *J. molec. Biol.*, **74**, 331 (1973).
- ²⁰ Perutz, M. F., *Proc. R. Soc.*, **B173**, 113 (1969).

(free enthalpies in Driessens's terminology) reported by Duff³. These latter values are open to question because their calculation was based on analytical data for systems which were not demonstrated to be at equilibrium and because analytical data were used to calculate ΔG_f° values for compounds that were not formed in the experimental systems.

We have prepared a series of solid solutions with degrees of fluoride substitution, x , estimated chemically, was 0, 0.13, 0.24, 0.41, 0.57, 0.81, 0.89 and 1; details about the preparation of these compounds, involving a very slow precipitation process, will be published elsewhere. The solid-solution nature of these samples was verified by crystallographic techniques. Thus, a linear decrease in the magnitude of the unit cell parameter, a , was obtained with increasing values of x . As shown below, however, the thermodynamic properties of these compounds are slightly different from those expected for an ideal solid solution and, as should be anticipated⁴, these small differences significantly affect their solubility behaviour.

We report here the solubility of these compounds obtained by equilibrating them for more than 1 month with a series of dilute phosphoric acid solutions at 37° C. For a given member of the series we can express its solubility product constant, K_x , as $K_x = (\text{Ca}^{2+})^5(\text{OH})^{1-x}(\text{F})^x(\text{PO}_4^{3-})^3$ in which parentheses indicate activities. The ionic activities in the liquid phase can be calculated by iterative procedures similar to those previously reported⁸. Introducing the definition of the ionisation constant of water, K_w , it can be shown that $\log(\text{H}^+)^3(\text{PO}_4^{3-}) = 1/3 \log K(x, w) - 5/3[\log(\text{Ca}^{2+})(\text{OH})^2 + x/5 \log(\text{H}^+)(\text{F})]$ in which $K(x, w) = K(x) K(x)^{(9+x)}$. Thus, a plot of the quantity $-\log(\text{H}^+)^3(\text{PO}_4^{3-})$ against the quantity $-x/5 \log(\text{H}^+)(\text{F}) - \log(\text{Ca}^{2+})(\text{OH})^2$ should yield a straight line with a slope of $-5/3$. The plots for the various compounds will be displaced depending on the value of x but the slopes should be the same. The results of the analyses of the aqueous phase after equilibration were used to obtain the plots shown in Fig. 1. It is apparent that the data yielded a series of straight lines with the anticipated slope, within experimental error. These results constitute verification of the equilibria between the solid and liquid phases involved.

In Table 1 we report the values of K_x for the various degrees of substitution. A polynomial fit to these data (degree of determination 0.95) shows a minimum in K_x for a degree of substitution of 0.56, implying a minimum in the ΔG_f° for such a composition. The calculations reported by Duff³ do not show a minimum. This minimum, experimentally determined, could be anticipated on the basis of the disordered structure^{5,6} of hydroxyapatite and the hydrogen bonding⁷ of the hydroxyl

Fluoridated Hydroxyapatite Solubility and Caries Formation

SINCE the early observations¹ on reduction of caries with increasing fluoride concentrations in drinking water, the tacit assumption was made that fluoride incorporated in dental enamel acts as a cariostatic agent. An appealing explanation for the possible mechanism involved is based on the nature of the enamel mineral of which hydroxyapatite (OHA), $\text{Ca}_5\text{OH}(\text{PO}_4)_3$, is considered to be the prototype. Replacement of fluoride for the hydroxyl would yield fluorapatite (FA) which has a lower solubility. Analyses of surface layers of enamel, however, show that the fluoride contents are far below the level expected for FA. It is relevant then to ascertain to what extent partial substitution of the fluoride for the hydroxyl group in HA affects its solubility.

In a recent publication² related to the anti-cariogenic effect of fluoride, Driessens reported calculated solubilities of the solid solutions $\text{Ca}_5(\text{OH})_{1-x}\text{F}_x(\text{PO}_4)_3$ of which hydroxyapatite and fluorapatite are the end members. The subscript x is the degree of substitution of fluoride for hydroxyl and its numerical value varies from 0 (for HA) to 1 (for FA). His calculations are based on the standard free energies of formation ΔG_f°

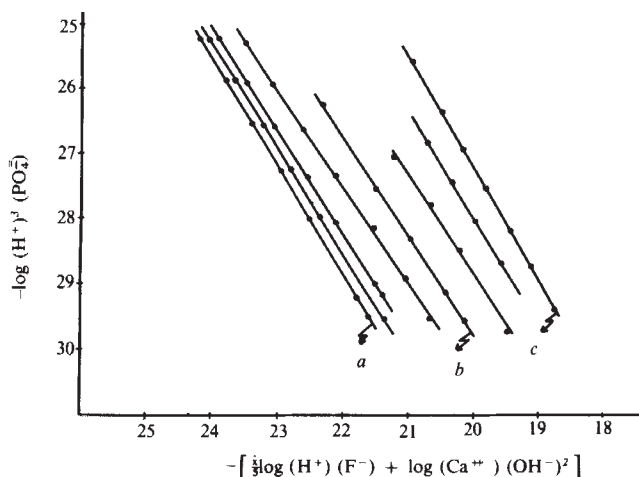


Fig. 1 Plots of quantities derived from solution compositions at equilibrium (see text). The slopes of the various lines (5/3 within experimental error) satisfy the equilibrium requirements. a, FA; b, $x=0.405$; c, OHA.

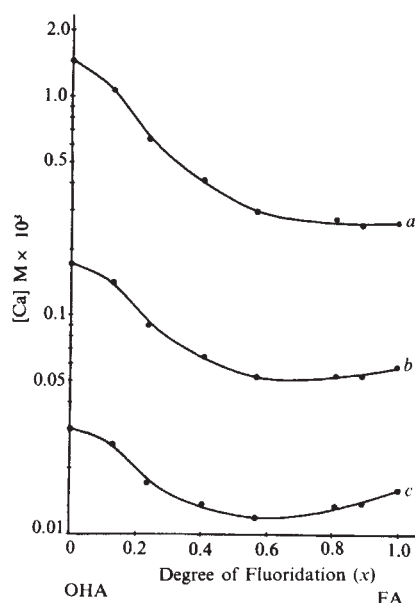


Fig. 2 Solubility of the solid solutions (expressed as molarity of calcium) as a function of the degree of fluoride substitution. Results are shown for, a, pH 5; b, pH 6; c, pH 7.

group to fluorine occurring in the structure. Both of these features would make their greatest contribution to the stability of the crystalline lattice at a degree of substitution of about 0.5.

Considering the constituent ions of the solid solutions plus the ion pairs⁸ $\text{CaH}_2\text{PO}_4^+$ and CaHPO_4^0 as the only species present in the equilibrated solution, we have constructed the plots shown in Fig. 2. On the ordinate are plotted the calcium concentrations in the aqueous phase maintained by the solid solution at equilibrium. Therefore, the curves reflect the variation of the solubility as a function of the degree of substitution. Values for x in the order of 0.05 are usually obtained for the surface enamel from fluoridated areas by assuming that the fluoride is uniformly distributed in the enamel sample. Contrary to the assertion by Driessens², the plots show that substitutions of this order (0.05) have very little effect on the solubility of hydroxyapatite. The fluoride concentration gradient is very steep^{9,10}, however, in the first few micrometres of enamel. Incidentally, this gradient is already established before tooth eruption; the fluoride concentrations in enamel from fluoridated areas are of a pre-eruptive origin¹⁰ and not as a result of topical effects as Driessens has suggested². In fact, recent work¹¹ on analyses of very shallow biopsy (0.2–0.4 μm) indicates that it is not unreasonable to consider that the degree of substitution at the very surface of the enamel may approach a value close to 0.5. If this is the case, our results indicate a significant reduction in solubility which undoubtedly could contribute to the cariostatic effect of fluoride. Important as this reduction in solubility may be in relation to caries prevention, there are other aspects that should be considered. For example, fluoride intake at early ages (while teeth are in the formative period) produces shallower pits and fissures in occlusal surfaces¹². Furthermore, it appears that

the electrochemical properties of the surfaces of fluoridated apatites are different from those of hydroxyapatite. Thus, it has been reported¹³ that the adsorption of salivary proteins is significantly different between these two kinds of compounds. We have verified this behaviour in our own laboratories. It is conceivable therefore that the selective formation of permselective coatings induced by the fluoridated surfaces of enamel may enhance the protection of the tooth structure.

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- Dean, H. T., Arnold, F. A., and Elvove, E., *Publ. Hlth Rep. Wash.*, **57**, 1155 (1942).
- Driessens, F. C. M., *Nature*, **243**, 420 (1973).
- Duff, E. J., *J. chem. Soc., A*, 1895 (1971).
- Moreno, E. C., in *International Symposium on Structural Properties of Hydroxyapatite and Related Compounds* (edit. by Young, R. A., and Brown, W. E.), chapter 15 (Gordon and Breach, New York, 1968).
- Kay, M. I., Young, R. A., and Posner, A. S., *Nature*, **204**, 1050 (1964).
- Young, R. A., and Elliott, J. C., *Archs oral Biol.*, **11**, 699 (1966).
- Van der Lugt, W., Knottnerus, D. I. M., and Young, R. A., *Caries Res.*, **4**, 89 (1970).
- Gregory, T. M., Moreno, E. C., and Brown, W. E., *J. Res. natn. Bur. Stand.*, **74**, A461 (1970).
- Aasenden, R., and Moreno, E. C., *Archs oral Biol.*, **16**, 1413 (1971).
- Isaac, S., Brudevold, F., Smith, F. A., and Gardner, D. E., *J. dent. Res.*, **37**, 318 (1958).
- Munksgaard, E. C., and Bruun, C., *Archs oral Biol.*, **18**, 735 (1973).
- Aasenden, R., and Peebles, T. C., *Archs oral Biol.* (in the press).
- Ericson, T., and Ericsson, Y., *Helv. odont. Acta*, **11**, 10 (1967).

Reversal by Narcotic Antagonist of a Narcotic Action elicited by a Conditional Stimulus

THERE is considerable evidence to indicate that learning is important in narcotic dependence¹. However, there has been little animal research into the mechanism of the interaction between environmental stimuli and drug action which maintains dependence. In morphine-dependent rats² and monkeys³ conditional stimuli previously associated with withdrawal can elicit physiological disturbances characteristic of narcotic withdrawal. Also, during withdrawal the conditional stimuli previously associated with morphine substitution can elicit blockade of morphine withdrawal⁴. In an experiment by Roffman *et al.*⁴, after a number of pairings with morphine injections, a neutral stimulus (bell) elicited a blockade of withdrawal hypothermia, a task normally accomplished by morphine itself. It was then suggested that there is an overlap between the brain mechanisms sensitive to morphine action and those affected by conditional stimuli⁵. Therefore, repeated pairing of hyperthermic effects of morphine with the stimulus-induced neural activity results in the conditioning of the former to the latter. We reasoned that if the brain activity due to a conditional stimulus evokes activity of the morphine receptors, a morphine antagonist should reverse the effectiveness of that stimulus in mimicking the morphine action. To obtain evidence in favour of this hypothesis we used naloxone, a specific antagonist of morphine action⁶.

Table 1 Values of K_x for Various Degrees of Substitution

Degree of substitution (x)	Solubility product constant (K_x)
0.0	$7.36 \pm 0.93 \times 10^{-60}$
0.13 ± 0.004	$2.13 \pm 0.11 \times 10^{-60}$
0.24 ± 0.01	$8.44 \pm 2.59 \times 10^{-62}$
0.41 ± 0.01	$2.32 \pm 0.81 \times 10^{-62}$
0.57 ± 0.02	$6.55 \pm 2.17 \times 10^{-63}$
0.81 ± 0.03	$5.87 \pm 1.30 \times 10^{-62}$
0.89 ± 0.03	$7.99 \pm 0.90 \times 10^{-62}$
1.00 ± 0.03	$3.19 \pm 0.14 \times 10^{-61}$

We used male Long-Evans rats (200–250 g) housed individually in a room maintained at 21–23° C with lights alternating on a 12 h dark-light cycle. Food (Purina chow) and water were available *ad libitum* except during injections and when physiological measurements were being made. The rats were given two equally spaced injections of morphine sulphate daily in increasing doses until a maintenance dose was reached. The first injection consisted of 10 mg kg⁻¹, which was increased by 10 mg kg⁻¹ every day until the tenth day, when each injection consisted of 100 mg kg⁻¹. The rats were maintained at this dosage (200 mg kg⁻¹ d⁻¹) for 3–5 d and then withdrawn. Each morphine injection was given in a well-lit room which was sound-attenuated and maintained at a constant temperature of 21° C. Each injection was paired with a bell which was turned on 1 min before the injection and turned off at the completion of that injection. After each injection, the rat was returned to its home cage away from the injection room.

During the withdrawal period of 24 h these rats were divided into three groups. One group (thirty-six rats) was not given any treatment during abstinence to obtain withdrawal hypothermia. A second group (six rats) was injected with morphine and exposed to the bell as during the addiction phase to serve as non-withdrawal controls. The third group (six rats) also continued to be injected and exposed to the bell as during the addiction phase. In this case, however, saline was injected instead of morphine sulphate to obtain the effect of conditioned stimuli during withdrawal from morphine. Thirty minutes after the last treatment, or after 24 h without treatment in the case of group 1, rectal temperature was measured by inserting a thermocouple probe 5 cm inside the rectum for 60 s. Following that measurement, the first group was divided into six subgroups (six rats each). One subgroup was then injected with naloxone (2 mg kg⁻¹), another with morphine (100 mg kg⁻¹), a third was exposed to the bell, and a fourth to the bell followed by naloxone given 10 min after the bell. The fifth group was given morphine followed by naloxone 30 min later. The last group was not given any treatment to serve as a control for withdrawal hypothermia.

It was previously established^{4,5} that the bell paired with saline does not affect rectal temperature in non-addicted rats. Also, naloxone did not cause any change in temperature of the non-addicted rats.

Table 1 Effect of Conditional Bell and Morphine on Rectal Temperature of Morphine-dependent Rats during Abstinence

Treatment during 24 h abstinence*	N	Rectal temperature (°C) † mean ± s.e.
None	36	37.42 ± 0.03
Bell and morphine ‡	6	39.06 ± 0.06
Bell and saline	6	38.06 ± 0.06

The rats were addicted by repeated injections of morphine paired with bell.

* After a maintenance dose of 100 mg kg⁻¹ (total daily dose of 200 mg kg⁻¹) the morphine injections were withheld for 24 h and one of the three treatments applied.

† Measured 30 min after the indicated treatment.

‡ Same dose as during maintenance (100 mg kg⁻¹).

Morphine injection reversed the withdrawal hypothermia and produced hyperthermia in these rats (Table 1) as was previously reported by others⁷. Exposure to the bell without morphine also reversed the withdrawal hypothermia as expected from our previous study^{4,5}. In a pilot experiment, presentation of the bell during abstinence or after morphine injection had not affected rectal temperature of a group of animals which had been repeatedly exposed to a bell during addiction phase, which was not regularly paired with morphine injections.

Data summarised in Table 2 show the effect of naloxone on the rectal temperature of the addicted rats undergoing

Table 2 Effect of Naloxone, Morphine or Conditional Stimulus Administered after an Abstinence Period of 24 h in Morphine-addicted Rats

Test treatment*	N	Rectal temperature§ mean (°C) ± s.e.	Change (°C)	P¶
None	6	37.00 ± 0.08	—	—
Naloxone †	6	36.68 ± 0.06	−0.32	<0.05
Bell	6	38.04 ± 0.06	+1.04	<0.001
Bell + naloxone	6	36.17 ± 0.06	−1.87	<0.001
Morphine ‡	6	38.87 ± 0.21	+1.87	<0.001
Morphine + naloxone	6	37.37 ± 0.26	−1.28	<0.001

* Given 24 h after last morphine injection.

† 2 mg kg⁻¹, given interperitoneally.

‡ Same dose as during the maintenance regime (100 mg kg⁻¹).

§ Measured 30 min after last treatment.

|| From their respective control given in third column. Naloxone, morphine or bell group is compared with none group, while bell + naloxone group is compared with bell-alone group and morphine + naloxone is compared with morphine-alone group.

¶ Student's *t* test, two tailed.

withdrawal. In these rats naloxone without any pretreatment produced slight hypothermia. However, when these rats were injected with naloxone after the bell, which itself increased rectal temperature by a degree, naloxone caused a severe decrease of that temperature. This effect of naloxone was similar to that seen after morphine injection in the addicted rats. Morphine caused marked hyperthermia in the abstinent rats. Naloxone injected after morphine caused a considerable decrease of temperature in these rats.

Either injection of morphine or exposure to the bell caused hyperthermia in the abstinent rats. The rectal temperature of morphine-treated rats was, however, higher than that of rats exposed to the bell. A quantitative similarity between morphine and the bell is not justified because we used a large dose of morphine (100 mg kg⁻¹) and attempted no variation of bell intensity for comparison.

Our findings on the ability of conditional stimulus to mimic the counter-withdrawal effect of morphine are in accord with earlier findings on conditioning of the agonistic effects of morphine. According to Bykov⁸, Devlov and Petrova in Pavlov's laboratory used physical stimuli to elicit conditional electrocardiographic changes associated with morphine injection. Collins and Tatum⁹ serendipitously discovered a conditional salivary reflex associated with morphine injection. Kleitman and Crisler¹⁰ replicated conditioning of salivary-reflex using morphine as the unconditional stimulus. It should be pointed out that our observations are limited to abstinence hypothermia and therefore our conclusions may not be applicable to other signs of abstinence. In rats, hypothermia has been considered one of the most reliable signs of abstinence.

Narcotic antagonists are known to precipitate narcotic withdrawal. As far as we know, however, interaction of narcotic antagonist with the learned conditional effects of morphine has not been previously studied. The ability of naloxone to block the physiological responses evoked by conditional stimuli in the same manner as it blocks the unconditional morphine effects has both theoretical and practical implications.

Of theoretical importance is the suggestion from this study that the conditional stimulus may evoke activity in the brain pathways specifically sensitive to the agonistic actions of morphine and to morphine dependence. Alternatively, the common belief that naloxone acts only by displacing morphine from its receptor may be questioned. Rather, naloxone may exert an agonistic influence on brain substrates originally insensitive to naloxone but rendered sensitive by the actions of morphine. Other experiments showing the inability of narcotic agonists to reverse actions of narcotic antagonists^{11–13} raise similar doubts about the

hypothesised mode of action of narcotic antagonists. A recent report on the naloxone antagonism of analgesia produced by brain stimulation¹⁴ supports this possibility of the direct action of naloxone.

The practical importance of this finding is related to the use of narcotic antagonists in the therapy of narcotic addiction. The current rationale behind the use of narcotic antagonists in the treatment of heroin addicts is that treatment with these drugs will result in the extinction of heroin consumption because of the blockade of the 'high' sought from agonistic effects of illicit heroin. Our data suggest that narcotic antagonists may also be valuable in extinguishing heroin habit associated with the conditional placebo effects of heroin-seeking behaviour. These effects have been considered to be major factors in relapse to the addiction in humans¹.

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- ¹ Wikler, A., *Behav. Sci.*, **16**, 92 (1971).
- ² Wikler, A., and Pescor, F., *Psychopharmacologia*, **10**, 235 (1963).
- ³ Goldberg, S., and Schuster, C., *J. exp. anal. Behav.*, **10**, 235 (1967).
- ⁴ Roffman, M., Reddy, C., and Lal, H., *Drug Addiction*, 1 (edit. by Singh, J., Miller, L., and Lal, H.), 223 (Future Publishing, New York, 1972).
- ⁵ Roffman, M., Reddy, C., and Lal, H., *Psychopharmacologia*, **29**, 197 (1973).
- ⁶ Martin, W., *Pharmacol. Rev.*, **19**, 463 (1967).
- ⁷ Martin, W., and Sloan, J., *Pharmacopsychiat. Neuro-psychopharmacol.*, **1**, 260 (1968).
- ⁸ Bykov, K., trans. by Gantt, W., *The Cerebral Cortex and the Internal Organs* (Chemical Publishing Co., 1957).
- ⁹ Collins, K., and Tatum, A., *Am. J. Physiol.*, **74**, 14 (1925).
- ¹⁰ Kleitman, N., and Crisler, G., *Am. J. Physiol.*, **79**, 571 (1927).
- ¹¹ Wikler, A., Fraser, H., and Isbell, H., *J. Pharmac. exp. Ther.*, **109**, 92 (1953).
- ¹² Wikler, A., and Carter, R., *J. Pharmac. exp. Ther.*, **109**, 92 (1953).
- ¹³ Cheney, D., Judson, B., and Goldstein, A., *J. Pharmac. exp. Ther.*, **182**, 189 (1972).
- ¹⁴ Mayer, D., Akil, H., and Liebeskind, J., *Fed. Proc.*, **32**, 693 (1973).

Survival of Common Bacteria in Liquid Culture under Carbon Dioxide at High Temperatures

MOLTON *et al.*¹ have reported that bacteria in liquid cultures survived exposure to normally lethal temperatures when heated in a high pressure CO₂ atmosphere. Because these results could influence planetary quarantine constraints, particularly for the planet Venus, and because no protection against thermal death by CO₂ has been reported previously, an attempt was made to verify their results.

In addition to the same strains of *Escherichia coli*, *Aerobacter aerogenes*, *Serratia marcescens*, and *Bacillus subtilis* used by Molton *et al.*¹, a thermophilic bacillus (YTG-2 S^r) and two genetically marked *E. coli* strains were tested. Initial experiments followed the methods for bacterial cultivation, CO₂ pressurisation, heating, and depressurisation described by Molton *et al.*¹, with the exception that YTG-2 S^r was grown at 65° C. Viability assays differed only in that dilutions beyond 10⁻⁶ were not used and duplicate rather than triplicate platings were made.

Stationary and log-phase cultures of *E. coli* B, *E. coli* K 12 S^r, *A. aerogenes*, YTG-2 S^r, *S. marcescens*, and *B. subtilis* were exposed to 160° C for periods of 3.5 to 22 h

Table 1 Effect of High Pressure CO₂ on the Survival of *E. coli* and *B. subtilis*

Organism	Pressure (atm)	Temperature °C	Time (h)	Initial (Viable counts ml ⁻¹)	Final	Survival (%)
<i>E. coli</i> B	9.5	22	1	2.7 × 10 ⁹	3.4 × 10 ⁹	125.9
<i>E. coli</i> B	19.7	22	1	2.7 × 10 ⁹	3.3 × 10 ⁹	122.2
<i>E. coli</i> B	19.7	15	18	2.7 × 10 ⁹	3.1 × 10 ⁹	114.8
<i>B. subtilis</i>	9.5	22	1	1.1 × 10 ⁸	1.2 × 10 ⁷	10.9
<i>B. subtilis</i>	9.5	22	1	1.4 × 10 ⁸	4.7 × 10 ⁷	33.6
<i>B. subtilis</i>	19.7	22	1	1.1 × 10 ⁸	3.5 × 10 ³	0.0032
<i>B. subtilis</i>	19.7	22	1	1.4 × 10 ⁸	1.9 × 10 ⁶	1.4
<i>B. subtilis</i>	19.7	15	18	1.1 × 10 ⁸	0	0

under 19.7 atm CO₂. No survivors were obtained. A less severe exposure to heat was also examined using stationary and log-phase cultures of *E. coli* B, *E. coli* B T-Pr-, and *B. subtilis*. No survivors were obtained with any of the three strains after exposure to 125° C for either 5 or 18 h under 9.5 atm CO₂ pressure.

Microscopic observation of *E. coli* B, YTG-2 S^r, *A. aerogenes*, and *S. marcescens* subjected to a high pressure CO₂ atmosphere heated at 125° or 160° C revealed that nearly 100% lysis had occurred with heating periods as short as 6 h. Cells of *B. subtilis*, however, did not lyse. The 30 min exhaust time used by Molton *et al.* (personal communication) was extended to 1 to 1.5 h in an attempt to reduce lysis. The slower exhaust rate neither increased survival nor reduced lysis significantly.

Since no survival was obtained at high temperatures and as the physical condition of heated cells did not improve with a very slow depressurisation rate, the effect of pressurisation and depressurisation without heating was examined. Cultures of *E. coli* B did not lyse or lose viability when exposed at room temperature (22° C) for 1 h at 9.5 or 19.7 atm CO₂ (Table 1). Exposure for 18 h at 15° C under 19.7 atm CO₂ did not decrease the viability of an *E. coli* B culture either. These results suggest that the pressurisation with CO₂ and subsequent depressurisation were not sufficient to kill unheated cells of *E. coli* B. In two separate experiments with late log-phase cultures of *B. subtilis*, survival decreased to 10.9% and 33.6% after 1 h exposure to 9.5 atm CO₂ at 22° C. At 19.7 atm CO₂, a 1 h exposure of *B. subtilis* cells at 22° C resulted in 0.0032% and 1.4% survival. No survivors were obtained when *B. subtilis* cultures were exposed to 19.7 atm CO₂ for 18 h at 15° C. Thus, with *B. subtilis*, high CO₂ pressure without heating was quite lethal.

In summary, we were unable to reproduce the results reported by Molton *et al.*¹ and suggest that additional studies are needed before conclusions can be reached regarding the thermal protection of bacteria by carbon dioxide at high pressures.

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- ¹ Molton, P., Williams, J., and Ponnampereuma, C., *Nature*, **243**, 242 (1973).

Production of Bladder Stones by Human T Mycoplasmas

SINCE the first isolation of human T mycoplasmas¹, their pathogenic role has remained controversial. These organisms, present in the urogenital tract of men and women, are unique among mycoplasmas in possessing urease². They have recently been isolated from other mammals³⁻⁵. Bovine strains have

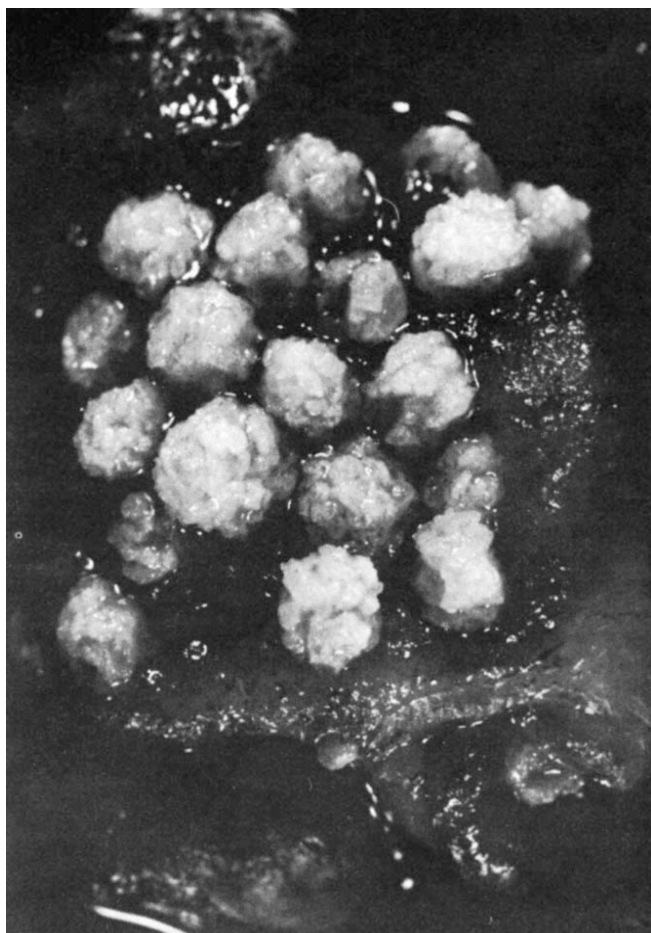


Fig. 1 Bladder stones from a rat killed 6 weeks after renal inoculation with T mycoplasma ($\times 3$).

caused experimental pneumonitis in calves⁶ and mastitis in cattle. In the latter experiment, human T mycoplasmas were unable to produce mastitis⁷. Human T mycoplasmas have been implicated as causative agents of non-gonococcal urethritis in men and pelvic inflammatory disease, puerperal infection and reproductive failure in women⁸. Their true relationship to human disease, however, remains unknown, and until now they have never been reported to produce disease in experimental animals. We now report the production of bladder stones during experimental infection of rats by human T mycoplasmas.

The T960 strain of human T mycoplasma was grown aerobically at 37° C in trypticase soy broth containing 15% horse serum, 1.25% yeast extract, penicillin (1,000 units ml⁻¹), 0.1% urea and 0.002% phenol red (pH 6.0). After overnight culture, an alkaline pH change occurred (final pH 7.0–7.1). The culture was centrifuged (20,000g for 30 min at 4° C) and concentrated 100-fold by resuspending in the supernatant giving 10⁶–10⁸ colour changing units (CCU) ml⁻¹. Male Sprague-Dawley rats (250–400 g) fed antibiotic-free chow were infected by injecting 0.1 ml into the bladder lumen or renal medulla. They were killed 6 weeks later and examined and cultured for bacteria as previously described⁹.

Bladder calculi developed in 78% of male rats after direct renal injection (Table 1). There was no difference in the incidence of stones when 10⁶ and 10⁸ CCU were used. No bladder stones formed after inoculation with concentrated T mycoplasma broth (pH 7.0) alone. The stones were 2–4 mm in diameter (Fig. 1), with up to thirty per rat. Crystallographic analysis showed all the bladder calculi to be essentially pure MgNH₄PO₄·6H₂O. All the eleven stones analysed were 100% MgNH₄PO₄·6H₂O except one, which contained 1% carbonate

apatite. Ten per cent of normal male and female rats of this strain form minute gravel deep in the renal pelvis. Such gravel was distinctly different from the bladder calculi which develop following T mycoplasma infection, as it was composed of calcium oxalate dihydrate and hydroxyapatite with no trace of MgNH₄PO₄·6H₂O. Renal gravel from two rats infected with T mycoplasmas was composed of pure MgNH₄PO₄·6H₂O. In none of the experiments with T mycoplasmas was there any effect on the incidence of renal gravel compared with uninfected controls.

Most rats with bladder stones had thickened bladders and dilated ureters. Hydronephrosis was present in one third of the rats. The kidneys showed tiny cortical scars with fibrosis and some round cell infiltration around the needle tract. Similar renal changes were seen following injection of broth only. No bladder thickening, dilated ureters or hydronephrosis was seen, however, in broth controls or rats injected with T mycoplasma which did not develop stones. There was no disease in the prostate or seminal vesicles. Urine, kidney and prostate cultures were negative for bacteria.

Stones formed in three of six rats as early as one week following renal inoculation. Female rats (200–220 g) failed to produce bladder stones (Table 1) and a similar sex difference has been noted in experimental stone formation¹⁰. Intravenous infection failed to produce stones; in males however, inoculation into the bladder lumen of males produced stones at the same high incidence as that seen following renal infection (Table 1).

While attempting to recover T mycoplasmas from the animals, we found a growth inhibitor for T mycoplasmas in renal homogenates similar to that described for large colony mycoplasma¹¹. This inhibitor was present at 10⁻¹ dilution but was absent on further dilution. No T mycoplasmas were detected in the urine, kidney or prostate of uninoculated rats. T mycoplasmas were isolated from urine and kidney cultures of rats which developed bladder stones following renal infection. A similar isolation rate was seen in infected rats which did not develop stones. Three of ten bladder stones washed extensively in phosphate buffered saline yielded T mycoplasmas on culture.

To determine whether viable organisms are necessary for stone formation, a group of rats was given erythromycin injections daily (80 mg kg⁻¹) for 7 d following renal inoculation with T mycoplasmas. This dose gave urine levels of erythromycin ten times the minimal inhibiting concentration for T960 *in vitro* (3.12 µg ml⁻¹). Erythromycin did not prevent stone formation (Table 1) although urine, kidney and prostate cultures were negative for T mycoplasmas. The implication that viable T mycoplasmas were not required for stone forma-

Table 1 Incidence of Bladder Stone Formation in Rats following Infection with T Mycoplasma

Group	Sex	Route of infection	No. of animals with stones/ Total No. in group
T mycoplasma	♂	Renal	49/63
Uninoculated	♂	Renal	0/17
T mycoplasma broth	♂	Bladder	0/8
T mycoplasma	♀	Renal	0/9
T mycoplasma	♂	Intravenous	0/10
T mycoplasma	♂	Bladder	19/21
T mycoplasma + erythromycin treatment	♂	Renal	7/10
T mycoplasma, acetone killed	♂	Renal	16/20
T mycoplasma 100° C, 15 min	♂	Renal	4/10
<i>P. mirabilis</i> in T mycoplasma broth 100° C, 15 min	♂	Renal	0/5
		Bladder	0/5
<i>M. hominis</i>	♂	Bladder	0/12

Rats were all killed 6 weeks after infection. Inocula for these experiments were as follows: T mycoplasma, 10⁶–10⁸ CCU per 0.1 ml; *P. mirabilis*, 10⁹ organisms per 0.1 ml; *M. hominis*, 10⁹ CCU per 0.1 ml.

tion was further examined by injecting rats with killed organisms. A culture of T mycoplasmas was centrifuged and resuspended in 33% acetone overnight. The organisms were then centrifuged and resuspended in complete T mycoplasma media (pH 7.1) to 0.01 of the original culture volume. Renal inoculation with 0.1 ml mycoplasmas killed in acetone produced bladder stones at the same high rate as with live T mycoplasmas (Table 1). Acetone treatment killed the T mycoplasmas and preserved minimal urease activity as measured by pH change. T mycoplasma killed by heat (100° C, 15 min) which had no urease activity still produced stones although at a lower incidence (Table 1) than organisms killed in acetone. Viable T mycoplasmas are thus not required for stone formation.

The observation that heat killed organisms caused stones suggested that T mycoplasmas might do so simply by providing a nidus. This was studied further with *Proteus mirabilis* which has a potent urease and causes $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ stones during urinary tract infection in rats¹². *P. mirabilis*, grown in trypticase soy broth was centrifuged and resuspended to 10^9 organisms per 0.1 ml in T mycoplasma broth and then killed by heating at 100° C for 15 min. No bladder stones formed after inoculation of dead *P. mirabilis* (Table 1). The failure of heat killed *P. mirabilis* to cause stones suggests that the nature of the particulate matter may determine whether it can serve as a nidus for calculus formation.

Another mycoplasma species was then examined for stone forming ability to see whether this was a property limited to T mycoplasmas. *Mycoplasma hominis*, the other principal human urogenital mycoplasma, was grown in T mycoplasma media with 1% L-arginine in place of 0.1% urea and the penicillin was omitted. None of twelve rats infected with 10^9 CCU per 0.1 ml developed vesical calculi (Table 1). Stone formation is thus not a general property of all mycoplasma species.

Our results establish the ability of human T mycoplasmas to cause vesical calculi in male rats following bladder or renal inoculation. Stones were not inhibited by antibiotics, and were produced by acetone or heat killed T mycoplasmas so that their formation does not require viable organisms. The specific association of $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ stones with infection by urease-producing bacteria has long been appreciated¹³. This would suggest that the urease content of T mycoplasmas may be a factor in stone generation. But $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ stones also occur experimentally in the bladder of rats in the absence of urea-splitting organisms¹⁴.

One of the unresolved aspects of stone formation is the site of nucleation and the subsequent retention of the stone nucleus within the urinary tract^{15,16}. The property of T mycoplasma colonies to adsorb mammalian cells¹⁷ may relate directly to this point. For example, T mycoplasmas may attach to epithelial cells in the urinary tract by specific receptors and serve as a stone nidus. As mycoplasmas seem to adsorb serum proteins to their surface¹⁸, T mycoplasmas may also be able to adsorb urinary mucoprotein, the principal component of the organic matrix of stones¹⁹. Further, aggregation of the Tamm-Horsfall urinary mucoprotein can be caused by lowering the urea concentration²⁰. Thus, the common presence of a T mycoplasma in the urinary tract, its capacity to attach to mammalian cells, and perhaps to adsorb proteins to its surface, and aggregate mucoprotein by breaking down urea, all suggest it may serve as a nucleus and part of the organic matrix of human urinary tract stones.

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Received August 20, 1973.

- ¹ Shepard, M. C., *Am. J. Syph. Gonorrhea vener. Dis.*, **38**, 113 (1954).
- ² Shepard, M. C., and Lunceford, C. D., *J. Bact.*, **93**, 1513 (1967).
- ³ Taylor-Robinson, D., Haig, D. A., and Williams, M. H., *Ann. N. Y. Acad. Sci.*, **143**, 517 (1967).
- ⁴ Tan, R. J. S., and Markham, J. G., *Jap. J. exp. Med.*, **41**, 247 (1971).
- ⁵ Taylor-Robinson, D., Martin-Bourgon, C., Watanabe, T., and Addey, J. P., *J. gen. Microbiol.*, **68**, 97 (1971).
- ⁶ Gourlay, R. N., and Thomas, L. H., *J. comp. Path.*, **80**, 585 (1970).
- ⁷ Gourlay, R. N., Howard, C. J., and Brownlie, J., *J. Hyg. Camb.*, **70**, 511 (1972).
- ⁸ McCormack, W. M., Braun, P., Lee, Y.-H., Klein, J. O., and Kass, E. H., *N. Engl. J. Med.*, **288**, 78 (1973).
- ⁹ Friedlander, A. M., and Braude, A. I., *J. infect. Dis.*, **126**, 645 (1972).
- ¹⁰ Gustafsson, B. E., and Norman, A., *J. exp. Med.*, **116**, 273 (1962).
- ¹¹ Kaklamanis, E., Thomas, L., Stanopoulos, K., Borman, I., and Boshwitz, C., *Nature*, **221**, 860 (1969).
- ¹² Braude, A. I., and Sieminski, J., *J. Bact.*, **80**, 171 (1960).
- ¹³ Brown, T. R., *J. Am. med. Assoc.*, **36**, 1395 (1901).
- ¹⁴ Vermeulen, C. W., Grove, W. J., Goetz, R., Ragins, H. D., and Correll, N. O., *J. Urol.*, **64**, 541 (1950).
- ¹⁵ Sutor, D. J., in *Urolithiasis: Physical Aspects* (edit. by Finlayson, B., Hench, L. L., and Smith, L. H.), 43 (National Academy of Sciences, Washington, DC, 1972).
- ¹⁶ Vermeulen, C. W., in *Urolithiasis: Physical Aspects* (edit. by Finlayson, B., Hench, L. L., and Smith, L. H.), 237 (National Academy of Sciences, Washington, DC, 1972).
- ¹⁷ Manchec, R. J., and Taylor-Robinson, D., *J. Bact.*, **100**, 78 (1969).
- ¹⁸ Sethi, K. K., and Brandis, H., *Med. Microbiol. Immun.*, **157**, 113 (1972).
- ¹⁹ Boyce, W. H., in *Renal Stone Research Symposium* (edit. by Hodgkinson, A., and Nordin, B. E. C.), 181 (Churchill, London, 1972).
- ²⁰ McQueen, E. G., and Engel, G. B., *J. Clin. Path.*, **19**, 392 (1966).

Genetic Dissection of Active Electrogenesis in *Paramecium aurelia*

NEUROPHYSIOLOGISTS frequently use various drugs and toxins to interfere with specific membrane mechanisms. A genetic approach can achieve the same results. In taking this approach to the problem of excitation it is desirable to have an organism, like *Paramecium*, on which both formal genetic¹ and orthodox electrophysiological² studies can be made.

The behaviour of ciliated protozoa is under the direct control of the membrane. Thus, we have efficient methods of screening for membrane mutants which rely on their behavioural peculiarities³⁻⁴. Wild-type paramecia show "avoiding reactions" to various stimuli⁵. This is accomplished through a period of backward swimming in which the direction of ciliary beat is reversed. This behavioural reaction is correlated with membrane excitation. Mechanical, electrical and chemical stimuli can result in depolarisation of the membrane^{6,7}. Suprathreshold depolarisation activates a regenerative increase in Ca conductance. The increase in internal Ca concentration through the Ca influx apparently activates the reversal of ciliary beat⁸. The system returns to normal through repolarisation and active ion transport.

The upstroke of the action potential is considered the result of a Hodgkin cycle, with Ca^{2+} instead of Na^{+} carrying the action current in *Paramecium*^{6,7}. Concepts such as Ca inactivation, K-delayed rectification, and ion pumping are also introduced to explain the relevant phenomena. Although the molecular mechanisms of these membrane functions in protozoa as in metazoa are still obscure, it is reasonable to think that only macromolecules, presumably membrane proteins, can carry out such complex functions. Genic mutations with corresponding loss of the normal gene products could therefore lead to malfunctions in various membrane processes, which in *Paramecium* are closely associated with the ciliary motion, and could eventually surface as behaviourally aberrant phenotypes.

Kung^{3,4,9} and more recently Chang and Kung^{10,11} have mutagenised populations of *P. aurelia* with N-methyl-N'-nitro-N-nitrosoguanidine and, by screening, have obtained

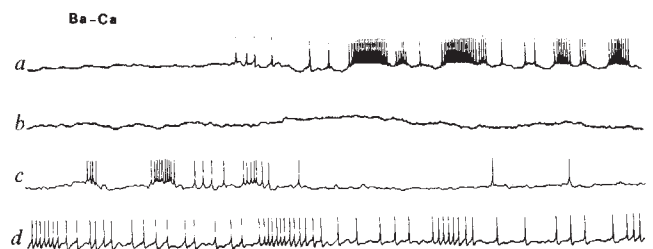


Fig. 1 Potential records of various strains 2-3 min after Ba solution entered the bath. *a*, Wild type; *b*, Pawn; *c*, Fast-2; and *d*, Paranoiic. Calibration: 5 s and 50 mV.

over 170 lines of mutants with various behavioural abnormalities indicating membrane malfunctions. Three unlinked genic mutants each representing a type were used here. These mutants were (1) "Pawns" (Stock d4-95, genotype *pwB pwB*), behaviourally characterised by the inability to reverse their ciliary beat even upon extreme stimulation. This behavioural deficiency was found to be correlated with the loss of proper membrane excitation¹²; (2) "Fast-2" (Stock d4-91, genotype *fna fna*) having an accelerated forward movement and an insensitivity specifically to Na stimulation; and (3) "Paranoiics" (Stock d4-90, genotype *Pa Pa*) which show a form of violent over-reaction to Na⁺ but not to other stimuli. The wild type Stock 51s (non-kappa bearing) of syngen 4 of *P. aurelia* was used as control.

Much of the literature on the behaviour of *Paramecium* describes the immediate reactions when cells from one solution are put into a second solution of different ionic composition^{13,14}. The bulk of the electrophysiological work in *Paramecium* deals, however, with the evoked potential changes when the cells have been equilibrated for a few minutes to bath solutions of various ionic compositions^{6,7}. To study the physiological correlates to chemotactic responses against various cations, we continuously monitored the membrane potential through various changes of ionic solutions.

The cell was first adapted in a K solution of 4 mM KOH, 1 mM CaCl₂, 1 mM citric acid and 1 mM Tris-HCl, pH 7.2. In this adaptation solution, membranes of wild type and all mutants were quiet and registered a resting potential of -20 to -30 mV.

To test the wild-type membrane reaction to Ba²⁺, a Ba solution of 4 mM BaCl₂, 1 mM CaCl₂ and 1 mM Tris-HCl, pH 7.2, was let into the bath to replace the K solution. The resting potential gradually diminished to -5 to 0 mV in 3 to 5 min. At this level the membrane spontaneously discharged. Such repetitive firings of all-or-none action potentials are known to be correlated with quick backward jerks observed in the free-swimming animals when they encounter the Ba solution².

In reaction to Ba solution, both Fast-2 and Paranoiic mutants generated the Ba spikes typical of the wild type (Fig. 1*a*, *c* and *d*). Considering the variations encountered within the same strain, the frequencies of these spikes observed in different strains are not meaningful. There was, however, a complete absence of action potentials in the case of Pawns treated identically with the other strains. Even under conditions known to increase the spike frequency and duration in wild type, such as a solution with Ca²⁺ concentration reduced to 0.3 mM, Pawn membrane never fired (Fig. 1*b*). This result agrees with the finding of Kung and Eckert¹² that Pawn is not capable of proper electrogenesis due to an impairment of the molecular mechanism for the voltage-sensitive Ca conductance changes. It also agrees with the behavioural observations that all strains discussed avoid Ba except Pawns³.

We discovered a distinct pattern of active electrogenesis correlated with the chemotactic avoiding reactions against Na⁺ by the wild type. This pattern appeared in 15 to 25 s after a Na solution (4 mM NaCl, 1 mM CaCl₂ and 1 mM

Tris-HCl, pH 7.2) began to flow into the bath in place of the K solution. No obvious change in the apparent resting level was observed, yet active depolarisations occurred. The rising phase of each episode began with a slow but sudden depolarisation rising from the resting level or rebounding from a slightly hyperpolarised level resulting from a previous episode. The rate of rise increased rapidly to that typical of the upstroke of action potentials. The spike peaked at about 20 mV from the resting level. The potential then oscillated for several hundred ms before returning to or slightly overshooting the resting level.

This gradually damped into patterns with lower spiking frequency, with spikes interspersed with spikeless depolarisations. Careful observation suggested that each spiked depolarisation episode was correlated with a sudden reversal of ciliary beat, and that in free swimming animals it most likely corresponded to the short avoiding reaction observed when the cell encountered Na⁺. This behavioural correlate, and the fact that 10⁻⁷ to 10⁻⁵ g ml⁻¹ of tetrodotoxin did not abolish the active electrogenesis, indicated that the action current was probably carried by Ca²⁺ although Na⁺ may play an important part in events previous to the spikes.

Fewer but active depolarisations were observed when Pawns encountered the Na solution (Fig. 2*b*). Each depolarisation episode differed, however, from that of the wild type (Fig. 2*a*) by a clear lack of spikes. The plateau magnitude and duration of this depolarisation and the kinetics of repolarisation did not differ significantly from the wild-type pattern. This mutated form of active electrogenesis related to no observable behavioural response and apparently caused no ciliary reversal.

No sign of active electrogenesis was observed when Fast-2 first encountered the Na solution (Fig. 2*c*). There was not even the slow rise of resting level found in the other strains in reaction to the Na solution. After a long delay (more than 5 min), however, the membrane eventually drifted to -10 mV. At this time, trains of all-or-none action potentials were observed. This membrane also showed spike-like hyperpolarisations much more frequently than normal membrane.

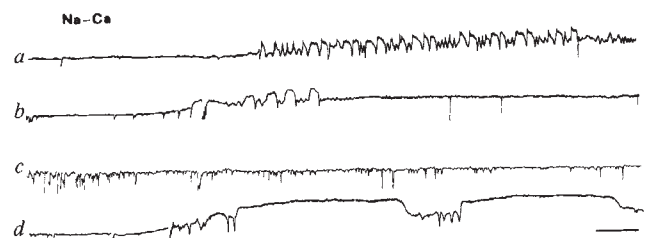


Fig. 2 Intracellular potential records during chemotaxis to Na⁺. Na solution (without citrate) was let into the bath in place of the adaptation solution at the beginning of traces *a*, *b* and *d*; *c* begins 30 s after the Na solution starts to flow into the bath. Calibration: 5 s and 50 mV.

The membrane of Paranoiic reacted to the Na solution with a peculiar pattern (Fig. 2*d*). There were active depolarisations but they were often sustained at the plateau for up to 60 s. Two such responses lasting around 20 s are shown in Fig. 2*d*. The kinetics of repolarisation is very different from that of wild type or Pawns. When the plateau phase of the depolarisation was shortened, the recovery kinetics closer to that of the wild type. As in wild type, there was also variation in the upstroke of the depolarisation. The prolonged action depolarisations coincided with the Paranoiic over-reaction to Na of this mutant. Details of the mutant electrical activities will be published elsewhere.

Mutations are usually deleterious, resulting in impairment of orderly biological processes. Thus, the mutant patterns

of electrogenesis in the presence of Na^+ could be viewed as modifications of the wild type pattern with single point blockages (Fig. 3).

The depolarisation pattern of Pawns can be viewed as the wild type pattern with a block on the upstroke of the action potential. Current injections of various strengths and durations up to 2×10^{-9} A for 1 s (depolarising the membrane by 20 mV) during our experiments in Na solution evoked no spikes. The nature of the spikeless depolarisation pattern observed in Pawns in reaction to the appearance of Na^+ is obscure. It is apparently triggered by Na^+ , never observed in the K-Ca or Ba-Ca solutions used and may relate to chemoreception of Na^+ .

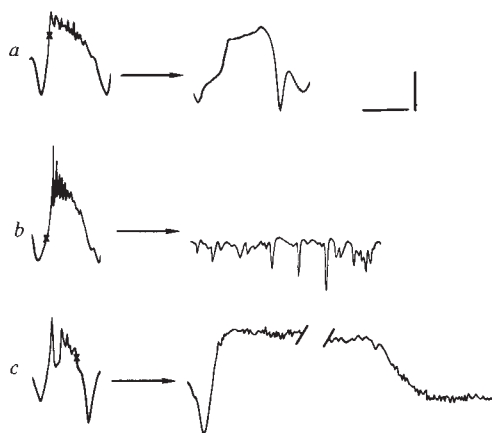


Fig. 3 A hypothesis showing that mutational blockages at various points of a normal depolarisation which occurs during Na chemotaxis can lead to different aberrant patterns. The three figures on the left are variations of the wild type patterns recorded in Fig. 2; the figures on the right are mutant patterns produced by blocks at different stages of a normal depolarisation. *a*, The spiking component is blocked by the *pw* gene leading to the Pawn pattern. *b*, Gene *fna* blocks the early depolarisation event and thus results in the Fast-2 pattern. *c*, Gene *Pa* blocks the proper repolarisation process and thus sustains and prolongs the depolarisation. Calibration: 1 s and 10 mV.

The pattern of Fast-2 in reaction to Na can be viewed as the result of a mutational blockage in the normal depolarisation process, located before the Pawn blockage. This results in a total loss of depolarisation correlating with the specific loss of chemotactic reactions against Na^+ . Presence of Ba spikes, delayed action potentials in Na^+ as well as electrically evoked action potentials indicates that the Fast-2 membrane is excitable. The mutational blockage, therefore, does not extend to the component blocked by the Pawn gene.

The intriguing patterns of Paranoiac in the Na solution may be viewed as the result of a genetic block of the normal process of repolarisation. Because of the lack of a proper shut-off mechanism related to Na^+ , the membrane potential stays near the peak of depolarisation for a greatly prolonged period and eventually drifts back to the resting level through a secondary repolarisation mechanism unaffected by this mutation.

As a description of the physiological abnormalities of various membrane mutants in *P. aurelia*, this study demonstrates the power of the genetic approach in systematically modifying a complicated biological phenomenon: the active electrogenesis during chemotactic reaction against Na^+ . This approach may aid in the search for a molecular mechanism for excitation by correlating the mutant phenotypic expression at various levels of investigation and by identifying the gene products. This rationale of applying genetics to neurobiology was first stated by Benzer¹⁵.

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- ¹ Sonneborn, T. M., in *Methods of Cell Physiology*, 4 (Academic Press, New York, 1970).
- ² Naitoh, Y., and Eckert, R., in *Experiments in Physiology and Biochemistry* (Academic Press, London, 1972).
- ³ Kung, C., *Z. Vergl. Physiol.*, **71**, 142 (1971).
- ⁴ Kung, C., *Genetics*, **69**, 29 (1971).
- ⁵ Jennings, H. S., *Behavior of Lower Organisms* (Indiana University Press, Indiana, 1906).
- ⁶ Eckert, R., and Naitoh, Y., *J. Protozool.*, **19**, 237 (1972).
- ⁷ Eckert, R., *Science, N.Y.*, **176**, 473 (1972).
- ⁸ Naitoh, T., and Kaneko, H., *Science, N.Y.*, **176**, 523 (1972).
- ⁹ Kung, C., in *Neural Organisms: Their Significance for Neurobiology* (Plenum Press, London, in the press).
- ¹⁰ Chang, S. Y., and Kung, C., *Science, N.Y.*, **180**, 1197 (1973).
- ¹¹ Chang, S. Y., and Kung, C., *Genetics* (in the press).
- ¹² Kung, C., and Eckert, R., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 93 (1972).
- ¹³ Dryl, S., in *Behaviour of Micro-organisms* (Plenum Press, New York, 1973).
- ¹⁴ Naitoh, Y., *J. gen. Physiol.*, **51**, 85 (1968).
- ¹⁵ Benzer, S., *Proc. natn. Acad. Sci. U.S.A.*, **58**, 1112 (1967).

Regulation of Spawning Behaviour in the Female Goldfish

ALTHOUGH the role of hormones in the control of sexual behaviour in female mammals is well established¹, this is not the case with fish². In spite of several reports that castration of female fish eliminates sexual behaviour², only two investigations have demonstrated successful steroid replacement therapy^{3,4}. We report here, however, the induction of sexual behaviour in female goldfish (*Carassius auratus*) by injection of ovulated eggs into the ovarian lumen of individuals with vitellogenic ovaries, and also by administration of both eggs and oestrogen to individuals with regressed ovaries.

In goldfish, ovulation can be induced in several days by a change in water temperature from 12° to 20° C. At this 'summer' temperature, females ovulate once or several times within about a month, after which the ovaries regress. At ovulation, the eggs are released into an ovarian cavity which is continuous with the oviduct. Oviposition (release of eggs through the ovipore) occurs during a series of spawning acts. The female approaches and enters floating vegetation in a head-up position, the male following from below. Both turn on their sides and, as they break the water surface, the male presses against the female and appears to push her out of the water. Eggs and sperm are released this time and fertilisation occurs. This procedure may be repeated up to several hundred times during 1–3 h. Spawning behaviour of the female is seen only in the presence of male goldfish and green plants⁵.

Encouraged by Yamazaki's⁵ findings that (1) spawning behaviour was seen only in goldfish which had ovulated, and (2) hand-stripping of ovulated fish terminated this behaviour, we investigated the role of ovulated eggs. We found that when oviposition is prevented by a plastic and glass plug in the ovipore of an ovulated female, spawning behaviour is extended. Whereas spawning usually lasts less than 2 h, 'plugged' females perform the spawning act (without oviposition) throughout the day of ovulation and sometimes continue the following day.

To determine whether placement of eggs in the ovarian lumen would induce, rather than prolong, spawning, ovulated females were allowed to spawn with a male for 20 min to obtain individual normal spawning rates. The fish were then

hand-stripped of all remaining ovulated eggs, placed with an actively courting male, and observed for 1 h to ensure that spawning had terminated. Then all were anaesthetised in MS222 (tricaine methanesulphonate, Sandoz) and given one of three treatments: (1) a handling control, (2) a plastic and glass oviduct plug fitted in the ovipore, and (3) an injection of the individual's own ovulated eggs through the ovipore, followed by an ovipore plug to prevent egg loss. In this last treatment, eggs were squeezed into a plastic cup and taken up in a 1 ml plastic syringe fitted with plastic tubing (PE 190). Eggs were injected into the ovarian lumen by inserting the tip of the plastic tube through the ovipore. It was important to prevent the eggs coming into contact with water, as this caused adhesion and hardening.

Within 10 min of treatment each female was placed with a sexually active male and behaviour recorded for 1 h. There was no difference between pretreatment spawning rates of the three groups, but injection of eggs stimulated spawning (Table 1).

Table 1 Effects of Injection of Ovulated Eggs into the Ovarian Lumen on Spawning Behaviour of Ovulated, Hand-stripped Goldfish

Group	N	Before treatment Spawning rate*			After treatment Induced spawning rate†		
		No. spawn- ing	Mean	Range	No. spawn- ing	Mean	Range
(1) Control	11	11	32	14-60	1	7	
(2) Oviduct plug	11	11	40	20-56	4	6.5	1-11
(3) Egg injected	11	11	34	9-60	11	28	7-57

* No. of spawning acts per 20 min.

† No. of spawning acts in the most active 20 min of the test hour.

Next, we sought a time after ovulation when egg injection did not induce spawning. Surprisingly, injection of ovulated eggs into females which had not ovulated for at least a month usually resulted in spawning within 2 h. Furthermore, physical substitutes for eggs (gelatine, petroleum jelly and ovipore plugs) sometimes induced spawning, but the response was much smaller than with egg injections, suggesting that chemical as well as mechanical stimuli may be involved.

Females kept at 20°-22° C for 4 months or longer invariably failed to respond to injected eggs. Their ovaries were small, resembling those of hypophysectomised fish, and probably represented the phenomenon of temperature regression found in *Gillichthys*⁶. The oocytes were in the first growth phase with little or no yolk.

We thought that the unresponsiveness of temperature-regressed fish may result from reduced oestrogen secretion. To test this, we investigated whether 17 β -oestradiol would restore the spawning behaviour of these fish. Four groups of female fish were used. Group 1 (control) received four or five saline injections on alternate days before being injected with eggs on the test day. Group 2 received oestrogen but was not given egg injections. Group 3 fish were injected with oestrogen before receiving eggs. Group 4 (recently-ovulated control) received only egg injections on the test day.

Fish in groups 1, 2 and 3 had regressed ovaries. In each group, half the females were known to have ovulated soon after being warmed to 20° C several months earlier; the others had not ovulated in that period. Fish of group 4 had ovulated 3-9 d before the test day. 17 β -Oestradiol was injected intraperitoneally as a saline suspension (0.6% NaCl, four drops Tween 80 per 100 ml) at a concentration of 2.5 μ g μ l⁻¹ and a dose of 20 μ g per g body weight on alternate days for 7 or 9 d. Control fish received either an equivalent volume of saline and Tween 80 (group 1) or no previous treatment (group 4). On the morning of the day after the final injection, females in groups 1, 3 and 4 were anaesthetised in MS222, injected with eggs (0.025 ml per g body weight) from an ovu-

Table 2 Effect of 17 β -Oestradiol on Spawning Behaviour of Temperature-regressed, Egg-injected Female Goldfish

Group ovaries	Treatment	N	No. spawning	Mean spawning acts per 3 h*
(1) Regressed	Saline and eggs	12	2	63
(2) Regressed	Oestradiol	6	0	0
(3) Regressed	Oestradiol and eggs	12	12	66
(4) Active	Eggs	7	7	94

* Based on responding individuals.

lated female, fitted with an ovipore plug, and placed in a 15 gallon test aquarium at 20° C. Group 2 was simply anaesthetised and left to recover in the test aquarium. An actively courting male was added to the aquarium after 30-50 min and the pairs were observed continuously for 3.5 h.

Of twelve control fish treated with saline and eggs, only two spawned (Table 2: group 1). The response was not measurably affected by whether the fish had ovulated since being held at 20° C (one of six spawned in each case). Oestradiol alone failed to induce spawning in the regressed females in group 2, again demonstrating the necessity of having eggs in the ovarian lumen. In contrast, spawning was observed in all regressed fish receiving oestradiol and eggs (group 3) and in all recently ovulated fish receiving only eggs (group 4). Our method for obtaining regressed female goldfish is, however, crude: some fish are not fully regressed and have ovaries in early stages of vitellogenesis. The two responding fish in group 1 were in this condition, suggesting that their spawning was due to endogenous steroids.

Yamazaki's⁵ and our own observations suggest that under natural conditions female goldfish have an extended breeding season during which they may ovulate and spawn several times during a period of weeks. An important role of oestrogen seems to be to induce and maintain responsiveness to the stimulus of ovulated eggs; there is no indication whether oestrogen acts peripherally or centrally, or both. The fact that readiness to perform oviposition behaviour in response to male courtship is induced by egg injection up to a month after the last ovulation indicates that the female goldfish is in an endocrine state appropriate for breeding for an extended period, but that spawning is normally synchronised with ovulation by the stimulus of a large intra-ovarian mass of ovulated eggs, which acts as a fine control in the timing of sexual responsiveness.

It would be interesting to determine whether comparable mechanisms exist in other fish or in other egg laying lower vertebrates. The fact that oestrogen alone restores sexual receptivity of female guppies which have been both hypophysectomised and gonadectomised⁴ suggests that in viviparous forms stimuli from the gonads or accessory structures may not be essential for the expression of sexual behaviour. In previous studies², the failure to induce sexual behaviour in female fish by oestrogen therapy may have been due to the absence of the requisite stimuli from internal sexual structures.

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¹ Davidson, J. M., and Levine, S., *A. Rev. Physiol.*, **34**, 375 (1972).

² Liley, N. R., in *Fish Physiology* (edit. by Hoar, W. S., and Randall, D. J.), **3**, 73 (Academic Press, New York, 1969).

³ Noble, G. K. L., and Kumpf, K. F., *Anat. Rec.*, **67**, Suppl. 113 (1936).

⁴ Liley, N. R., *Gen. Comp. Endocrinol.*, Suppl. 3, 542 (1972).

⁵ Yamazaki, F., *Mem. Fac. Fish. Hokkaido Univ.*, **13**, 1 (1965).

⁶ De Vlaming, V. L., *Comp. Biochem. Physiol.*, **44**, 697 (1972).

Evidence for the Two-state Model of Nematode Behaviour

A MODEL for the swimming and orientation of *Panagrellus silusiae* based on transitions between two behavioural states has been proposed¹. The basis of this model is that in normal, unstimulated conditions the nematode follows a more or less linear path with the anterior and posterior ends of the animal sweeping through a minimal area. This is termed the 'normal' behavioural state. When stimulated by chemical or physical means, the animal enters the 'activated' state, making rapid changes in orientation with the ends of the animal sweeping through a maximal area. In the case of a gradient of an attractant, the animal remains in the activated state until it orientates to the gradient by sensing the maximum difference in intensity of the stimulus between the posterior and anterior sensory structures, at which point the animal returns to the normal behavioural state and migrates to the source of attractant. Laser microbeam studies indicated that the copulatory spicules of the free-living *P. silusiae* function as the posterior receptors for orientation to mating attraction in this species¹, and a sensory function has also been found for the spicules in other species².

Ward³ proposed that chemotaxis of the free-living *Caenorhabditis elegans* is mediated by comparisons of stimuli by the anterior sensory receptors at successive lateral sweeps (klinotaxis). This mechanism requires some type of short-term memory system in nematodes, which has been demonstrated in *P. silusiae*¹.

The 'two-state' model was based on frame-by-frame analysis of video recordings of *P. silusiae* swimming in response to a range of chemical and physical stimuli, but the klinotaxis model³ was based on examination of the tracks of normal and mutant nematodes in response to gradients of cyclic nucleotides, ions, amino acids and pH. Here we report further support for the two-state model based on analysis of tracks of *P. redivivus* males migrating in gradients of sexual attractant.

Tracks of individual males of *P. redivivus*, strain N, on 4% Czapek dox agar were recorded photographically (N. Croll, personal communication). The sources of mating attraction were blocks of 1.5% agar exposed to females for 24 h (ref. 4). Males were placed 2.5 cm from the source of attractant and tracks were recorded after 15 min. Analysis of the tracks involved measuring the linear distance travelled, the number of turns (changes in direction of tracks), loops (360° turns accomplished through forward motion only) and reversals (backward motion) made in the 15 min period (Table 1).

Table 1 Activities of Adult Males of Strain N *P. redivivus* in Unstimulated Conditions and in Response to Mating Attractant from Females of Strains C and N

Stimulus	Distance (cm)	Loops (No.)	Reversals (No.)	Turns (No.)
None	1.71 ± 1.06	5.43 ± 1.61	22.81 ± 3.16	26.59 ± 4.44
C females	2.13 ± 0.19	16.59 ± 3.31	34.42 ± 3.96	128.79 ± 14.47
N females	2.36 ± 0.42	15.30 ± 3.83	47.00 ± 9.44	150.00 ± 29.86

Figures are means during a 15 min test period.

Movement to attractant involves no significant increase in the distance travelled but does show significant increases in the number of loops, reversals and turns relative to those of males moving without attractant. This shows that the behavioural activities of males responding to sex attraction are different from the normal behaviour. The pattern of the tracks of males responding to sex attractant (Fig. 1a) is exactly what would be predicted by the two-state model.

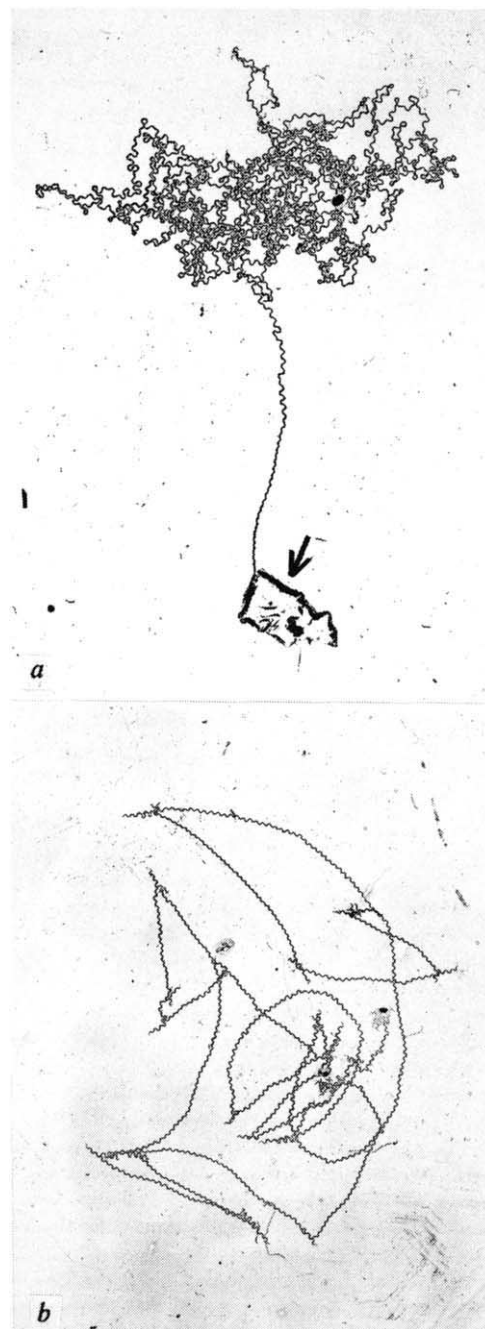


Fig. 1 Tracks of *P. redivivus* males, a, in response to sex attractant, b, unstimulated. The male responding to attractant (arrow) first undergoes a period of rapid changes in orientation (activated behaviour) and then migrates directly to the source of attractant.

Initially the males make rapid, constant changes in their orientation, with increased turning, looping and reversal; this is the period of activated behaviour. As the animal migrates towards the attractant the path is linear and direct; this is normal behaviour. Males moving when no attractant is present show a more or less linear pattern (Fig. 1b) broken by widely spaced changes in direction.

The two-state mechanism for sexual attraction does not preclude the functioning of klinotaxis in other classes of nematode chemotaxis. In *P. silusiae* sexual attraction is limited to one stage of the life cycle of one sex⁴, but non-specific food attraction occurs at all stages of both sexes. This latter attraction does not involve the two-state mechanism.

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¹ Samoiloff, M. R., McNichol, P., Cheng, R., and Balakanich, S., *Expl. Parasit.*, **33**, 253 (1973).

² Lee, D. L., *J. Zool., Lond.*, **169**, 281 (1973).

³ Ward, S., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 817 (1973).

⁴ Cheng, R., and Samoiloff, M. R., *Can. J. Zool.*, **49**, 1443 (1971).

Calcium Carbonate in Termite Mounds

THE presence of appreciable quantities of calcium carbonate in termite mounds on non-calcareous soil has intrigued pedologists for many years. Milne¹, for example, found a termite mound with 7% calcium carbonate and estimated that it contained about 2 t of calcium carbonate excluding the hard limestone (53% CaCO₃) base of the mound. The soil below the base of a termite mound may also be calcareous. The soil underneath one termite mound in an area of non-calcareous soil was found to have a mean of 1.7% calcium carbonate to a depth of 6 m, or about 20 t of calcium carbonate².

The task of finding the primary methods of calcium carbonate accumulation has been hindered by the fact that termite mounds are complex systems involving biological and physical processes. Milne¹ and Pendleton³ suggested that the most probable biological method was the collection of food by termites and formation of calcium carbonate from the mineralised residues. Other biological methods of calcium carbonate accumulation that have been advanced are as follows: (1) exchangeable calcium in soil collected incidentally by termites with their food⁴; (2) exchangeable calcium in soil collected purposely by termites requiring an alkaline environment⁵; (3) calcareous material collected from below the depth of soil said to be non-calcareous^{3,7}; and (4) calcium-containing groundwater brought up by termites². The physical methods proposed all involve evaporation of water containing calcium bicarbonate from a termite mound as the means of accumulating calcium carbonate, but they differ in the source and mode of entry of water into the mound. Thus Milne¹ suggested that water moves upward into the termite mound by capillary action. Den Doop⁴ considered that the water evaporating from the mound was derived from surrounding land in the wet season. Boyer² too describes how water from perched water tables drains laterally and collects underneath mounds of *Macrotermes subhyalinus*. Finally, Hesse⁵ found an association between calcareous *Macrotermes* mounds and poorly drained soil which led him to state that saturation of the base of a termite mound by groundwater was a prerequisite of calcium carbonate accumulation.

The early ideas on the mode of accumulation of calcium carbonate in termite mounds were considered by Pendleton³ who said of some of them that they "seem too fantastic to repeat". He came to the conclusion that there was no known method by which termites or pedological processes could bring about the observed accumulation of calcium carbonate in termite mounds. There seems therefore to be a need for a different approach to the problem. A weakness of all proposals made hitherto is the long time that it would take to accumulate large amounts of calcium carbonate.

I have constructed a hypothesis that could account for more rapid accumulation of calcium carbonate in a termite mound.

The hypothesis (Fig. 1) is that calcium carbonate can accumulate in termite mounds by means of a two-stage process.

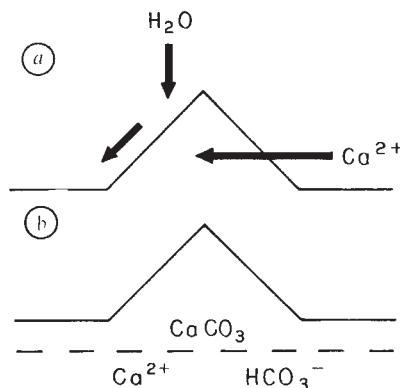
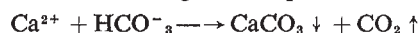


Fig. 1 Two stages in the accumulation of calcium carbonate in a termite mound. *a*, Elevation of the pH of the termite mound. This is brought about by termites carrying vegetation into a mound, aided by the fact that a mound sheds rainwater; *b*, precipitation of calcium carbonate as a result of contact between a termite mound of high pH and groundwater of low pH_c.

(1) Elevation of the pH status of the termite mound above that of the pH_c of the groundwater. An increase in the pH of a mound is brought about by termites importing vegetation which is decomposed to release exchangeable bases. Retention of exchangeable bases in the termite mound is facilitated by a low degree of leaching because a mound sheds water⁸.

(2) Saturation of the base of the termite mound by groundwater containing calcium bicarbonate which precipitates as calcium carbonate according to the equation



The tendency for calcium carbonate to precipitate from irrigation water on contact with soil is a well known alkalinity hazard of irrigation schemes, and it has been found by Bower¹ to be related to a modified Langelier saturation index:

$$\text{Modified saturation index} = \text{pH}_s - \text{pH}_c$$

where pH_s is the pH value of the soil and pH_c is the theoretical pH value that the water would have if in equilibrium with calcium carbonate. Bower states that precipitation of calcium carbonate occurs if the index is positive. Moreover, the percentage of the applied bicarbonate that precipitated was found to be highly correlated with the value of the index.

This pH-dependent precipitation of calcium is related to the presence of bicarbonate and carbonate ions and does not occur with other common anions in groundwater, namely, chloride and sulphate. Formation of calcium carbonate in termite mounds in some regions and not in others may therefore, perhaps, be explained by differences in the anion composition of the groundwaters.

The rate of accumulation of calcium carbonate by means of the pH-bicarbonate process outlined here could be relatively fast in situations where groundwater of low pH_c flows past the base of a *Macrotermes* mound of high pH value. Consequently, if this hypothesis is correct, it will be unnecessary to assume that termite mounds have existed for the thousands of years that it would take to accumulate large amounts of calcium carbonate by evaporation of water.

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¹ Bower, C. A., in *Proc. UNESCO Arid Zone Symposium, Teheran, Iran, 1958*, 215 (UNESCO, Paris, 1961).

² Boyer, Ph., in *La Vie dans les Sols* (edit. by Pesson, P.), 279 (Gauthier-Villars, Paris, 1971).

³ Burt, B. D., *J. Ecol.*, **30**, 125 (1942).

⁴ Den Doop, J. E. A., *E. Afr. agric. J.*, **4**, 89 (1938).

⁵ Hesse, P. R., *J. Ecol.*, **43**, 449 (1955).

⁶ Milne, G., *J. Ecol.*, **35**, 192 (1947).

⁷ Pendleton, R. L., *Thai. Sci. Bull.*, **3**, No. 2, 29 (1941).

⁸ Watson, J. P., *J. Soil Sci.*, **13**, 46 (1962).

⁹ Watson, J. P., *J. Ecol.*, **57**, 441 (1969).

book reviews

New horizons

Cosmology Now. Edited by Laurie John. Pp. 168. 8 plates. (BBC: London, November 1973.) £2.75.

THIS is, without doubt, the best introduction to modern cosmology available in book form. The list of contributors, each of whom provides one or two chapters, reads like a role of honour of the young cosmologists who have been at the forefront of the revolution in the subject over the past twenty years, with the addition of a couple of 'grand old men' such as Professor W. H. McCrea (whose outlook is certainly as youthful as that of any of the other contributors). For once, a claim made in the cover blurb (quoting Sir Bernard Lovell's introduction) can be taken at face value: this really is "the best description of the universe which can be given today".

But lest it seem that I am in the pay of the book's editor I should, perhaps, elaborate on the areas where it falls short of perfection, in the hope that some of these shortcomings might be rectified in a later edition. There is one glaring omission—in a book which is sure to attract the casual reader, and to whet his appetite for more cosmological fare, there is no bibliography or guide to further reading. Compared to this, the other points to which I take exception are only minor irritations, largely due, it would seem, to the editor's unfamiliarity with astronomy. Laurie John produced the radio series from which the book derives, and no doubt it is fitting that he should provide the introductory paragraphs which head each chapter. But surely among the wealth of cosmological talent contributing to the book someone could have checked these paragraphs, and the captions to the illustrations, for astronomical howlers?

Reference to quasars as "star like objects that may lie near the edge of the universe" is certainly misleading without qualification; far worse is the caption to Plate 8: "The star arrowed is a quasar"! It also seems a shame to introduce Martin Rees solely as "Plumian Professor of Astronomy" when the rest of his title ("and Experimental Philosophy") relates so appositely to the content of the book in general and his own contribution in particular. Apart from the quote from Sir Bernard Lovell the cover blurb is

irritatingly out of key with the book, dragging in a reference to "the 'black hole', that ever-growing celestial maelstrom" which is likely to cause further distress to those astronomers already infuriated by the extravagant popularisations of black hole astronomy which have appeared recently; they can, however, safely ignore the words on the cover and be guided as to the true nature of the book by Professor Roger Penrose, who points out that no astronaut is likely to encounter a black hole by accident—"they would have to seek out a black hole deliberately if they wished to experience this 'ultimate trip'". My final objection is purely a personal one—I have no liking for Professor John Taylor's homespun cosmological philosophy. But that may indicate a failing in me rather than in him.

Such a small catalogue of complaints shows, perhaps, how good the book is in general. As far as the detailed exposition of cosmological ideas is concerned there is little to say here except that all the contributions are readable and intelligible without any prior mathematical knowledge, and the few illustrations are equally clear and provide a valuable addition to the text. Quite apart from the 'introductory text' aspect of this volume, however, the contributions provide a valuable insight into the personalities of the contributors and the kind of bric-a-brac of odd bits of information which make it valuable in historical terms. I was particularly intrigued, for example, to discover from Dennis Sciama's contribution that Dicke and his colleagues were unaware of Gamow's earlier 'hot big bang' model of the Universe when they reintroduced the idea in 1965; I would have thought every astronomer remembered Gamow's work, if only because of his cunning choice of co-authors in writing the classic paper by Alpher, Bethe and Gamow—certainly I knew of this work in 1965, when much to my delight I discovered that paper as part of an undergraduate project. And certainly once discovered it is never forgotten.

Professor J. V. Narlikar also produces a memorable sentence: "Until the observational situation clarifies further it is premature to write off the steady state theory". That is particularly encouraging for those, like myself, who have a fondness for the steady state theory, and it does seem that the model has regained some ground since the

dark days of the mid-1960s. But I must confess that while my gut reaction is still 'steady state, right or wrong' my head tells me that the evidence in favour of the hot big bang is very nearly overwhelming.

I also have a fondness for Mach's Principle, first propounded by Bishop Berkeley and today, it seems, rather more widely approved than steady state cosmologies. And when investigating the triumph of head over gut in the big bang—steady state dilemma, the future historian of science will no doubt be intrigued to learn from Sciama's contribution that he was persuaded, in 1966, of the great difficulties presented by trying to explain radio observations in steady state theory terms by "my student, Martin Rees". That same Martin Rees contributes to the book in his capacity now as Plumian Professor; an indication of just how much ground has been covered by essentially one generation of cosmologists.

So the book is well suited to the casual reader, to the historian of science, to specialist astronomers and to students. With an eye particularly to the needs of the latter, I hope that it may soon be available in paperback form at an even lower price, although it is certainly good value already.

JOHN GRIBBIN

Descriptive embryology

Embryology and Phylogeny in Annelids and Arthropods. By D. T. Anderson. Pp. xiv+495. (Pergamon: Oxford and New York, October 1973.) £8.

THE human brain, it would seem, is well adapted to the recognition and analysis of two-dimensional patterns, corresponding to retinal images, but is, as all teachers of anatomy know, far less adept at dealing with three-dimensional configurations. In embryonic development we are confronted with three-dimensional patterns changing in time; to produce a total image of such a development as a whole would be equivalent to thinking in four dimensions. To go even beyond this, in seeking to analyse a comparison between the embryonic developments of a whole series of animal species would in effect add even more dimensions to the problem. The daunting difficulty of this goes far to explain the recent unpopularity of studies in comparative descrip-

tive embryology. Professor Anderson's book is to be welcomed as a bold and substantial inroad on this difficult field, comparable with Dawydoff's (1928) *Traité d'Embryologie Comparée des Invertébrés* or Johannsen and Butt's (1941) *Embryology of Insects and Myriapods*. The work is not merely a compilation from published sources, but incorporates much original work of Professor Anderson.

In seeking to draw phylogenetic conclusions from studies of embryology, the older authors looked for elements of Haeckelian "recapitulation", that is embryonic features resembling those of adults of more or less remote ancestral forms. Many features described by Anderson might be considered as recapitulatory in this sense; for example, the pre-mandibular somites and appendage rudiments of insect embryos, the transient coelomic sacs of numerous Arthropod embryos, the trilobite-like features of Crustacean nauplii and of the newly hatched *Limulus*, and the widely separated paired primordia of the insect labium. Most recent embryologists, Professor Anderson among them, minimise or ignore this line of evidence for ancestry.

Discounting recapitulation, he tries to draw phylogenetic conclusions from comparisons of overall developmental patterns in different taxa. In practice, of course, the limitations of humanity oblige him to concentrate attention on certain features abstracted from the total process; cleavage patterns, formation of germ layers, somite differentiation, are discussed in particular detail. Much stress is laid on 'fate maps' in relation to early embryonic stages. In this connection no distinction is made between 'mosaic' types of development, as in typical spiral cleavage of Annelida, where the future development of every cell is fixed from the beginning of cleavage, and 'regulation' types, known, for example, in many insects, where for a considerable time pieces detached from the embryo can be made good from the rest of it. The book gives well illustrated accounts of typical developmental patterns of all the major groups of Annelida and Arthropoda, but several phylogenetically interesting minor ones, such as the Echiuroidea (whose true position is probably in Annelida), the Tardigrada and Pentastomida, Myzostomida and Pycnogonida, for which embryological information is available, are omitted altogether from consideration.

The phylogenetic conclusions drawn by Professor Anderson coincide almost exactly with those expressed in a recent paper by Dr S. M. Manton "Arthropod Phylogeny—A Modern Synthesis" (*J. Zool., Lond.*, **171**, 111, 1973), to whom Anderson pays handsome tribute and dedicates his book. Dr Manton in turn

frequently cites Anderson in support of her own, more dogmatically expressed views. A central conclusion of both authors is thus expressed by Manton: "It used to be supposed that the Arthropoda were monophyletic . . . now the polyphyletic nature of the group is inescapable, as is the acceptance of parallel evolution of roughly similar structures, for instance, compound eyes and biting jaws, in unrelated groups, such as insects and crabs". Dr Manton splits the Arthropoda into three groups, Uniramia (Onychophora, Myriapoda, Hexapoda), Crustacea and Chelicerata, which she treats as independent phyla; Anderson goes even further in questioning whether these three groups could even have had a common ancestor within Annelida. Dr Manton consigns the Trilobita to a kind of limbo outside her system, remarking also that "We can do no fossil embryology". If embryology is restricted to development within the egg, this may be true, but Anderson devotes a good deal of space to post-hatching stages in Polychaeta and Crustacea, and the palaeontologists have traced developmental stages in Trilobita back to a stage apparently comparable with a nauplius. The "modern synthesis" completely ignores evidence from recent protein studies, for example, of lactic dehydrogenases and similar enzymes, or from moulting hormones and other such substances.

It seems likely to me that future evidence will offer little support to the phylogenetic ideas of either author; Professor Anderson's book will, however, retain its value for embryologists, on account of the good and detailed accounts it provides of the earlier development of such a large and important group of animals, as well as for the excellent, full and up to date bibliographies which are appended to each of his chapters. If Dr Manton's phylogenetic theories are eventually proved wrong, this will not detract from the value of her earlier work on the movements and coordination of arthropod appendages, and her latest paper should at least act as an intellectual challenge to those who disagree with her.

R. A. CROWSON

Salomon's house

Science and Politics. By Jean-Jacques Salomon. Translated from the French by Noel Lindsay. Pp. xxii + 277. (Macmillan: London and Basingstoke, July 1973.) £5.50.

THE science policy concerns of the 1960s are here paraded in full academic robes. The author was a Professor of Philosophy in Paris before he became head of the Science Policy Division of the Organisation for Economic Co-operation and Development, and his approach is that of a scholar: he gives no quick

tips on how to provide the manpower, organise the research institutes or run an international project, but he is well read and his views are always interesting and worth considering.

Tackling his subject historically, he sees science policy as "the child of war", the military model being the ideal one for the mobilisation of science. When he discusses policy for science, he never tires of denying that the rationality of science somehow rubs off on scientific affairs, including technological forecasting. For readers in England he need not have repeated the point so often—the danger of a take-over by quantitative methods has never been great here, partly perhaps because of the healthy innumeracy of our decision-makers. Alvin Weinberg's criteria for scientific choice and the associated debate in *Minerva* get the space they deserve on account of their intellectual interest as well as the criticism they deserve for being "non-operational".

On the matter of science in policy, Salomon rejects the notion of an apolitical elite and insists, with R. Gilpin, that it is impossible for scientists to free themselves from values and non-technical assumptions. He introduces the term "technonature" to denote the borderland between science and government, between knowledge and power; it is "the inevitable meeting ground between the ideology and the scientificity of science" and it "brutally teaches scientists that informing political decision is not the same thing as forming it".

There is an interesting historical inaccuracy regarding the author's eponymic house. In Salomon's house as conceived by Francis Bacon in *New Atlantis*, the last of the nine categories of workers, the Interpreters of Nature, "raise the former discoveries by experiments into greater observations, axioms and aphorisms". J.-J. Salomon says their function is "turning experiment into action" (page 8). Trivial in itself, the shift from axioms to action is indicative of one of his leading ideas: that one can "no longer" separate pure from applied research, theory from practice. But when in history was there a time when science was "as innocent and pure as on the first day"? Is this anything more than another Adam-and-Eve myth? Galileo, it should be remembered, made money by selling a "military compass".

Salomon seems to suggest that science lost its innocence around the time of the Second World War—as though the birthright of the Mertonian norms (disinterestedness and so on) was then sold for a mess of pottage. This view is more convincing as a description of ontogeny than of phylogeny; it applies quite clearly to individual scientists such as Oppenheimer but much less

clearly to science as a whole. Salomon even implies (page 183) that competition for recognition has become intense only since the 1920s. I suspect he misattributes the historical transformation: it is not so much the character of science which has changed as the writing about science which has become more sophisticated and realistic.

He is not critical enough of the discovery push type of thinking which sees technological innovation as the result of pure research. Historians—even non-Marxist ones—have realised that it is at least as illuminating to analyse the technological roots of science as the scientific roots of technology, and work on modern industrial innovation indicates that today, too, need pull is more important than discovery push and that the inputs from science into technology are in fact rather tenuous.

By the time a book appears in a second language, three years after its original publication, it should be free of historical howlers such as the one about "the steam engine, of which Watt had the first idea as early as 1765" (page 27). Readers used to the down-to-earth directness of the best English writings on the subject may find that too many of the Franco-English sentences need rereading before they yield a meaning. F. R. JEVONS

Making the best of it

Optimum Structures. By W. S. Hemp. Pp. xii+123. (Clarendon: Oxford; Oxford University: London, May 1973.) £7.50 boards; £3.50 paper covers.

In recent years a great deal of attention has been focused on optimal or optimum structures. Such systems have their components so arranged as to minimise internal forces and so dimensioned as to maintain uniform stresses. Originally this field of study proved to be of special interest to aircraft designers, but recently civil and structural engineers have also turned their attention to it. The basic principle of optimisation applies to all classes of structures, and the recent trend towards improved design of structures of minimum weight obtained at a minimum cost gathers momentum.

To achieve these aims a detailed knowledge of various factors and a clear picture of their mutual interdependence are required. Optimisation can produce the desired result and it can be described as a procedure used by the designer to decide on the one specific solution in a defined set of possible alternatives that will best satisfy a selected criterion. In the past the optimisation of structures has required lengthy and time-consuming calculations.

The advent of digital computers is changing this, enabling the designer to compare many solutions in his search for the optimum structure. At the same time the interest grows in the fundamental philosophy regarding optimum structures.

Although there are already several papers published on this topic, Professor Hemp's book is unique, not only because it deals at great length with the basic principles of the analysis of optimum structures, but also because it incorporates a great deal of fundamental and highly original work of the author. Whereas most of the published articles deal with numerical methods, this book is mainly concerned with the derivation of the general principles of optimum structures using linear programming and the calculus of variations. It covers the treatment of pin-jointed frameworks, beams, circular sandwich plates, Michell's structural continua and plates loaded in their planes. Optimisation of structures can very easily become highly sophisticated and extremely difficult depending on the number of constraints or independent parameters considered in the analysis.

Although the treatment by nature is highly mathematical, the author has succeeded in producing a lucid exposition of the up-to-date methods of determining structures of least volume of material. He pays special attention to the use of Lagrangian multipliers. This is a book which many research workers and some designers will find extremely valuable in their search for the ideal structural form.

Z. S. MAKOWSKI

Currents in insulators

The Theory of Electrical Conduction and Breakdown in Solid Dielectrics. By J. J. O'Dwyer. (Monographs on the Physics and Chemistry of Materials.) Pp. ix+317. (Clarendon: Oxford; Oxford University: London, September 1973.) £10.

ONE might think that the subject of 'currents in insulators' was a contradiction in terms. This is not so: insulators have a wide forbidden band and hence often block the transport of current. But while it takes effort to introduce carriers into the conduction band, once they are there they run down potential slopes in the conventional way unless arrested by imperfections. Because of the difficulties of experimental study, the theory of charge transport in dielectrics is not so advanced as in semiconductors and metals. Injection of charge into thin-film insulators is, however, now seen to be potentially useful and this should raise the level of research performed.

The other technologically relevant field is the one treated in this book:

when insulators are stressed to a very high field (over 10^8 V m⁻¹), new phenomena occur which turn the material into a good conductor, though perhaps only over a limited part of the area exposed to stress. This part then carries an increasing current until the material overheats and is destroyed. In high-voltage technology, these effects must be minimised where possible. For this, one must understand the conduction phenomena occurring at the onset of breakdown. The object of this book is to lay out, in a concise manner, the various pieces of theory and research concerning conduction in insulators which aid this understanding and then to build on these concepts a picture of the complex events leading to the final catastrophic breakdown.

The author has, on the whole, achieved these limited aims with a commendable economy of space. After a useful initial survey chapter, which sets the scene well, the reader is led, with little deviation, through the several types of conduction possible in insulators. The research results of many workers are quoted for cases in which it is advisable to show how a particular mathematical model is embodied in real life. So far, so good; however, the author is a specialist in the field of breakdown phenomena (this book is actually a greatly broadened form of a 1964 book outlining his own theory of breakdown). Perhaps because of this, he seems to lean over backwards to be impartial and, as a result, withholds any overt judgments on the relative merits of the experiments and theories described. Not only does he seem to be saying "now judge for yourself", but one is also left with the erroneous impression that every model ever devised for conduction in insulators is equally true and common, even though some do, in fact, conflict. It is a pity that the benefits of a highly informed point of view were so clearly withheld.

There is thus little to disagree with in this book. It is a good, dispassionate record of published fact. A few omissions can, however, be sensed by scanning the author and subject indexes; there is no entry for 'photoinjection' or 'filamentary conduction', although both loom large in charge transport through thin-film insulators—a field which one would expect to be covered in a book of the aims stated. Neither are the investigations of Berglund, Powell or Nicollian on various types of injection into silicon dioxide films, or the work of Dearnaley or Harrop on filamentary and a.c. conduction phenomena, given any mention. In general, the book thus has a bias towards 'bulk' experiments and places little reliance on thin films as media for elucidating transport phenomena—an unfortunate blind spot, since Lynch has recently built on such

work to predict successfully the breakdown strength of silicon dioxide.

With this exception, it must be said that for a research student or working scientist who wishes to be quickly and smoothly introduced to or updated with respect to the concepts of transport in insulators (ionic as well as electronic), it will be well worth his while to have this book on his shelf. A lot of effort has been expended in whittling down the arguments to their core and selecting the best experimental examples for the reader's inspection. If the reader then has to go on a bit further in order to make his own value judgments, he will still have saved himself a lot of time in the library. A. G. HOLMES-SIEDLE

Fermi surfaces

The Fermi Surface: Its Concept, Determination, and Use in the Physics of Metals. By A. P. Cracknell and K. C. Wong. Pp. xii+565. (Clarendon: Oxford; Oxford University: London, August 1973.) £15.

In his original work in 1926 Fermi introduced the thermodynamic potential, or degeneracy temperature—what we now call Fermi level—of a gas of electrons. Though he was well aware that these ideas were relevant to electrons in metals, he could hardly have foreseen what central role this concept would assume in solid-state physics and particularly in the theory of metals. As the preface to this book reminds us, Professor Mackintosh thinks that the most appropriate definition of a metal is "a solid with a Fermi surface", the surface being the locus of points in momentum space at which the one-electron energy is equal to the Fermi level. This quantum concept, in conjunction with the consequences of crystal periodicity, provides the foundation on which the properties of metals can best be discussed. The beautiful, and sometimes bizarre, shapes of these surfaces (the monster, the coronet, the super-egg . . .) are natural examples of abstract art, almost reflecting unconscious archetypal models.

The work which has led to the determination of these surfaces is among the most ingenious, elegant and fascinating in modern physics and this book aims at giving a complete authoritative account of all its aspects. It is a combination of an introductory treatise and an extended review article. The first two chapters give those parts of band theory which are necessary for a proper understanding of both theoretical and experimental methods. Chapter 3 describes the experimental methods, then two chapters, bringing up to date the well known review articles by one of the authors, describe the Fermi surfaces of metallic elements. Chapter 6 describes in an elementary way the effect

on Fermi surfaces of electron interactions, and the work ends with an admittedly perfunctory treatment of alloys.

Probably the most striking characteristic of this exposition is its extreme clarity. The treatment of even difficult points flows effortlessly and should be well within the grasp of graduates who know the fundamentals of solid-state physics and of quantum theory (old style—the Dirac notation is not used). Of course the task of giving a simplified treatment which is not partly misleading is an impossible one, but we come near to achieving it here, and many teachers will get valuable hints for their lectures. By and large the choice of material appears very sensible; all the necessary definitions and explanations are given (or almost all: for instance the definition of Clebsch-Gordan coefficients seems to be missing); the usual "it can be proved that" is always coupled to an appropriate reference. Some sections are perhaps unduly discursive and some elementary items are included which could have been assumed to form part of the background, for example, Einstein's and Debye's theories of lattice specific heats. A notable omission is any reference to liquid metals. At least the extent of blurring of the Fermi surface might have been indicated, and a reference to Professor March's monograph given.

The book is thus a very useful introduction to the subject, but its main value is as a collection of results of work on Fermi surfaces complete up to 1971 with some later references. The total number of references is almost one thousand and they all fit neatly into their appropriate slot. I have failed to find any aspect of any importance which is not adequately covered. The authors will receive the heartfelt gratitude of all "Fermiologists" and it is safe to say that this will remain the standard treatise on Fermi surfaces for a long time, even if some parts should become surpassed. It is a pity that the price should be so high, even taking into account today's wild inflation, and the quality of reproduction of the illustrations is not outstanding.

L. PINCHERLE

Gas theory

Mathematical Theory of Transport Processes in Gases. By J. G. Ferziger and H. G. Kaper. Pp. xiii+579. (North-Holland: Amsterdam and London, 1972.) Dfl. 120; \$35.25.

THE year 1972 was the hundredth anniversary of the publication of the Boltzmann equation—apparently an auspicious year for the publication of a book on the kinetic theory of gases as based on this famous equation. This theory gives a complete prescription for calcu-

lating transport properties of dilute gases from knowledge of the intermolecular forces. As such, it is one of the most elegant and highly-developed physical theories available. But any book published in 1972 must face a severe test—comparison with two well known monographs, *The Mathematical Theory of Non-Uniform Gases* by Chapman and Cowling, now in its third edition (1970), and *Molecular Theory of Gases and Liquids* by Hirschfelder, Curtiss and Bird, in its second printing (1964). These are rightly regarded as classics, and have set a standard by which all other books in the field are measured. Ferziger and Kaper have attempted the difficult task of combining the best features of both of these works in a textbook for graduate study. Let me quote their own words for the reasons for the attempt. Chapman and Cowling ". . . is rather difficult as a text and does not emphasise the calculation of transport properties sufficiently. . . . Hirschfelder, Curtiss and Bird, while very good in discussing the calculation of transport properties, compresses the theory far more than desirable".

In my opinion the authors have succeeded admirably in their aim. There is no doubt that a real need exists for such a book, as evidenced by a prevailing opinion to the effect that ". . . reading Chapman and Cowling is like chewing on broken glass; reading Hirschfelder, Curtiss, and Bird is like punching a feather pillow". I wish a book like this had existed when I first started to try to understand kinetic theory.

This book covers much the same ground as Chapman and Cowling, but with more emphasis on intermolecular forces and the computation of transport coefficients. There are also excursions beyond the range of the Boltzmann equation to include modern work on dense gas theory and some discussion of rarefied gas dynamics. A book like this cannot hope to be complete, and in some areas will become out of date very quickly. One can see some signs of this already happening, but I do not feel that this detracts appreciably from the book's value. The discussion of fundamentals and mathematical developments is so well done as to be of almost permanent value, and the treatments of the more rapidly changing areas will enable the reader to proceed for himself into the research literature.

Finally, it is interesting to note that the book developed from a course intended for aeronautical, mechanical and chemical engineers. It should be of equal value to physicists and chemists, and even to biologists interested in phenomena describable by Boltzmann-type equations. I predict that it will be a standard text for some time to come. E. A. MASON